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Lower Gastrointestinal Microbiota in Health and Irritable Bowel Syndrome: Characterisation and Effect of Probiotic Intervention

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ACADEMIC DISSERTATION

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Abbreviations

ACE	abundance-based coverage estimator
ANOVA	analysis of variance
BMI	body mass index
bp	base pair
CD	Crohn's disease
CDAD	*Clostridium difficile* associated diarrhoea
cfu	colony forming unit
DGGE	denaturing gradient gel electrophoresis
DNA	deoxyribonucleic acid
FISH	fluorescent *in situ* hybridisation
FOS	fructooligosaccharide
GI	gastrointestinal
GOS	galactooligosaccharide
IBD	inflammatory bowel disease
IBS	irritable bowel syndrome
IBS-A	alternating symptom subtype irritable bowel syndrome
IBS-D	diarrhoea-predominant irritable bowel syndrome
IBS-C	constipation-predominant irritable bowel syndrome
IBS-M	mixed symptom subtype irritable bowel syndrome
IL	interleukin
NEC	necrotising enterocolitis
NSAID	non-steroidal anti-inflammatory drug
OTU	operative taxonomic unit
PAR-2	proteinase-activated receptor two
PCA	principal component analysis
PCR	polymerase chain reaction
PI-IBS	post-infectious irritable bowel syndrome
QOL	quality of life
qPCR	quantitative real-time polymerase chain reaction
RDP	ribosomal database project
rRNA	ribosomal ribonucleic acid
SIBO	small intestinal bacterial overgrowth
TGGE	temperature gradient gel electrophoresis
TLR	toll-like receptor
T-RFLP	terminal-restriction fragment length polymorphism
TRAC	transcript analysis with the aid of affinity capture
UC	ulcerative colitis
16S rRNA	16S ribosomal ribonucleic acid
%G+C	percentage of guanine plus cytosine

Abstract

The human gastrointestinal (GI) microbiota is a complex ecosystem that lives in symbiosis with its host. The growing awareness of the importance of the microbiota to the host as well as the development of culture-free laboratory techniques and computational methods has enormously expanded our knowledge of this microbial community. Irritable bowel syndrome (IBS) is a common functional bowel disorder affecting up to a fifth of the Western population. To date, IBS diagnosis has been based on GI symptoms and the exclusion of organic diseases. The GI microbiota has been found to be altered in this syndrome and probiotics can alleviate the symptoms, although clear links between the symptoms and the microbiota have not been demonstrated. The aim of the present work was to characterise IBS related alterations in the intestinal microbiota, their relation to IBS symptoms and their responsiveness to probiotic theraphy.

In this thesis research, the healthy human microbiota was characterised by cloning and sequencing 16S rRNA genes from a faecal microbial community DNA pool that was first profiled and fractionated according to its guanine and cytosine content (%G+C). The most noticeable finding was that the high G+C Gram-positive bacteria (the phylum *Actinobacteria*) were more abundant compared to a corresponding library constructed from the unfractionated DNA pool sample. Previous molecular analyses of the gut microbiota have also shown comparatively low amounts of high G+C bacteria. Furthermore, the %G+C profiling approach was applied to a sample constructed of faecal DNA from diarrhea-predominant IBS (IBS-D) subjects. The phylogenetic microbial community comparison performed for healthy and IBS-D sequence libraries revealed that the IBS-D sample was rich in representatives of the phyla *Firmicutes* and *Proteobacteria* whereas *Actinobacteria* and *Bacteroidetes* were abundant in the healthy subjects. The family *Lachnospiraceae* within the *Firmicutes* was especially prevalent in the IBS-D sample.

Moreover, associations of the GI microbiota with intestinal symptoms and the quality of life (QOL) were investigated, as well as the effect of probiotics on these factors. The microbial targets that were analysed with the quantitative real-time polymerase chain reaction (qPCR) in this study were phylotypes (species definition according to 16S rRNA gene sequence similarity) previously associated with either health or IBS. With a set of samples, the presence or abundance of a phylotype that had 94% 16S rRNA gene sequence similarity to *Ruminococcus torques* (*R. torques* 94%) was shown to be associated with the severity of IBS symptoms. The qPCR analyses for selected phylotypes were also applied to samples from a six-month probiotic intervention with a mixture of *Lactobacillus rhamnosus* GG, *L. rhamnosus* Lc705, *Propionibacterium freudenreichii* ssp. *shermanii* JS and *Bifidobacterium breve* Bb99. The intervention had been previously reported to alleviate IBS symptoms, but no associations with the analysed microbiota representatives were shown. However, with the phylotype-specific assays applied here, the abundance of

the *R. torques* 94% -phylotype was shown to be lowered in the probiotic-receiving group during the probiotic supplementation, whereas a *Clostridium thermosuccinogenes* 85% phylotype, previously associated with a healthy microbiota, was found to be increased compared to the placebo group.

To conclude, with the combination of methods applied, higher abundance of *Actinobacteria* was detected in the healthy gut than found in previous studies, and significant phylum-level microbiota alterations could be shown in IBS-D. Thus, the results of this study provide a detailed overview of the human GI microbiota in healthy subjects and in subjects with IBS. Furthermore, the IBS symptoms were linked to a particular clostridial phylotype, and probiotic supplementation was demonstrated to alter the GI microbiota towards a healthier state with regard to this and an additional bacterial phylotype. For the first time, distinct phylotype-level alterations in the microbiota were linked to IBS symptoms and shown to respond to probiotic therapy.

List of original publications

This thesis is based on the following publications:

I Krogius-Kurikka L, Kassinen A, Paulin L, Corander J, Mäkivuokko H, Tuimala J & Palva A. Sequence analysis of percent G+C fraction libraries of human faecal bacterial DNA reveals a high number of *Actinobacteria*. BMC Microbiology 2009 Apr 8;9:68.

II Krogius-Kurikka L, Lyra A, Malinen E, Aarnikunnas J, Tuimala J, Paulin L, Mäkivuokko H, Kajander K & Palva A. Microbial community analysis reveals high level phylogenetic alterations in the overall gastrointestinal microbiota of diarrhoea-predominant irritable bowel syndrome sufferers. BMC Gastroenterology 2009 Dec 17;9:95.

III Malinen E, Krogius-Kurikka L, Lyra A, Nikkilä J, Jääskeläinen A, Rinttilä T, Vilpponen-Salmela T, von Wright A & Palva A. Association of symptoms with gastrointestinal microbiota in irritable bowel syndrome. World Journal of Gastroenterology 2010 Sep 28;16(36):4532-40.

IV Lyra A, Krogius-Kurikka L, Nikkilä J, Malinen E, Kajander K, Kurikka K, Korpela R & Palva A. Effect of a multispecies probiotic supplement on quantity of irritable bowel syndrome-related intestinal microbial phylotypes. BMC Gastroenterology 2010 Sep 19;10(1):110.

The publications are referred to in the text by their Roman numerals. All have been published in peer-reviewed open-access journals.

1. INTRODUCTION

Immediately after birth, microbes start to colonise the human gastrointestinal (GI) tract, followed by a lifelong symbiosis with the host (Camp *et al.*, 2009). As a result, the adult GI microbiota forms a taxonomically complex and ecologically dynamic ecosystem with up to 10^{14} microbes in total, the main phyla in colon being low %G+C Gram-positive *Firmicutes*, high %G+C Gram-positive *Actinobacteria* and Gram-negative *Bacteroidetes* and *Proteobacteria* (Andersson *et al.*, 2008; Turnbaugh *et al.*, 2009). Most of the bacteria present are strict anaerobes, which are difficult to culture, and the approaches to study the diversity of this community are thus culture-free (Zoetendal *et al.*, 2008). As the GI tract is exposed to this vast number of microbes, disturbances in the microbiota affect individual health, and vice versa (Sekirov *et al.*, 2010).

Up to a fifth of the Western population suffers from irritable bowel syndrome (IBS), which is a functional bowel disorder that impairs the quality of life (QOL) (Longstreth *et al.*, 2006). The term IBS refers to the symptoms, which include pain or discomfort associated with alterations in bowel habits (Longstreth *et al.*, 2006). Multifactorial aetiologies have been proposed for the syndrome, altered gut microbiota being one of these factors (Malinen *et al.*, 2005). Probiotics have shown a beneficial effect in many gastrointestinal conditions, including IBS, and the alleviation of IBS symptoms has been demonstrated with many probiotic strains and mixtures (Spiller, 2008).

In the research of this thesis, the GI microbiota of control subjects devoid of GI symptoms and patients with diarrhea-predominant IBS (IBS-D) was investigated by applying a method that enabled the segregation of the microbial community DNA according to its genomic %G+C content, followed by a sequencing approach to characterise the IBS-D-associated microbiota. The associations between intestinal symptoms and GI microbiota in IBS and samples from a six-month probiotic intervention trial were also assessed. The GI mirobiota analyses were conducted by using phylotype (species definition according to 16S rRNA gene sequence similarity) specific quantitative real-time PCR (qPCR) assays previously associated with IBS or health.

2. REVIEW OF THE LITERATURE

2.1 Gastrointestinal (GI) microbiota

2.1.1 Development of the GI microbiota

From birth onwards, a complex microbiota begins to colonise the human GI tract (Favier *et al.*, 2002; Koenig *et al.*, 2011) (Figure 1). The microbiota remains dynamic and resilient throughout adulthood (Biagi *et al.*, 2010). The delivery mode is crucial, as babies born by Caesarean section have a different colonisation pattern from those delivered through the birth canal (Dominguez-Bello *et al.*, 2010). The next main environmental factor affecting the colonisation pattern is the diet, as babies fed breast milk babies have a different species profile within their GI microbiota compared to formula-fed babies (Roger & McCartney, 2010). Delivery via the birth canal and breast feeding are both associated with higher counts and more diverse populations of bifidobacteria (Favier *et al.*, 2002; Eggesbø *et al.*, 2011).

The GI microbiota enters a third fundamental modification stage during weaning (Kurokawa *et al.*, 2007), and typically by one year of age it reaches a more stable and diverse stage (Palmer *et al.*, 2007). High subject specificity and relative stability over time are characteristic of the GI microbiota through adulthood (Vanhoutte *et al.*, 2004; Rajilić-Stojanović *et al.*, 2009). In the elderly, the microbiota becomes less diverse (Enck *et al.*, 2009b; Rajilić-Stojanović *et al.*, 2009; Claesson *et al.*, 2010). Eventually, the microbiota becomes significantly altered in centenarians (Biagi *et al.*, 2010). In addition, the genotype (Turnbaugh *et al.*, 2009) as well as environmental factors such as geographical origin (Li *et al.*, 2008), diet (Muegge *et al.*, 2011), weight (Turnbaugh *et al.*, 2009), health status (Sekirov *et al.*, 2010), antimicrobial medication (Jakobsson *et al.*, 2010), and the consumption of probiotics (Kajander *et al.*, 2008) and prebiotics (Flint *et al.*, 2007) may affect the composition of the GI microbiota throughout life.

2.1.2 Ecology and composition of the GI microbiota

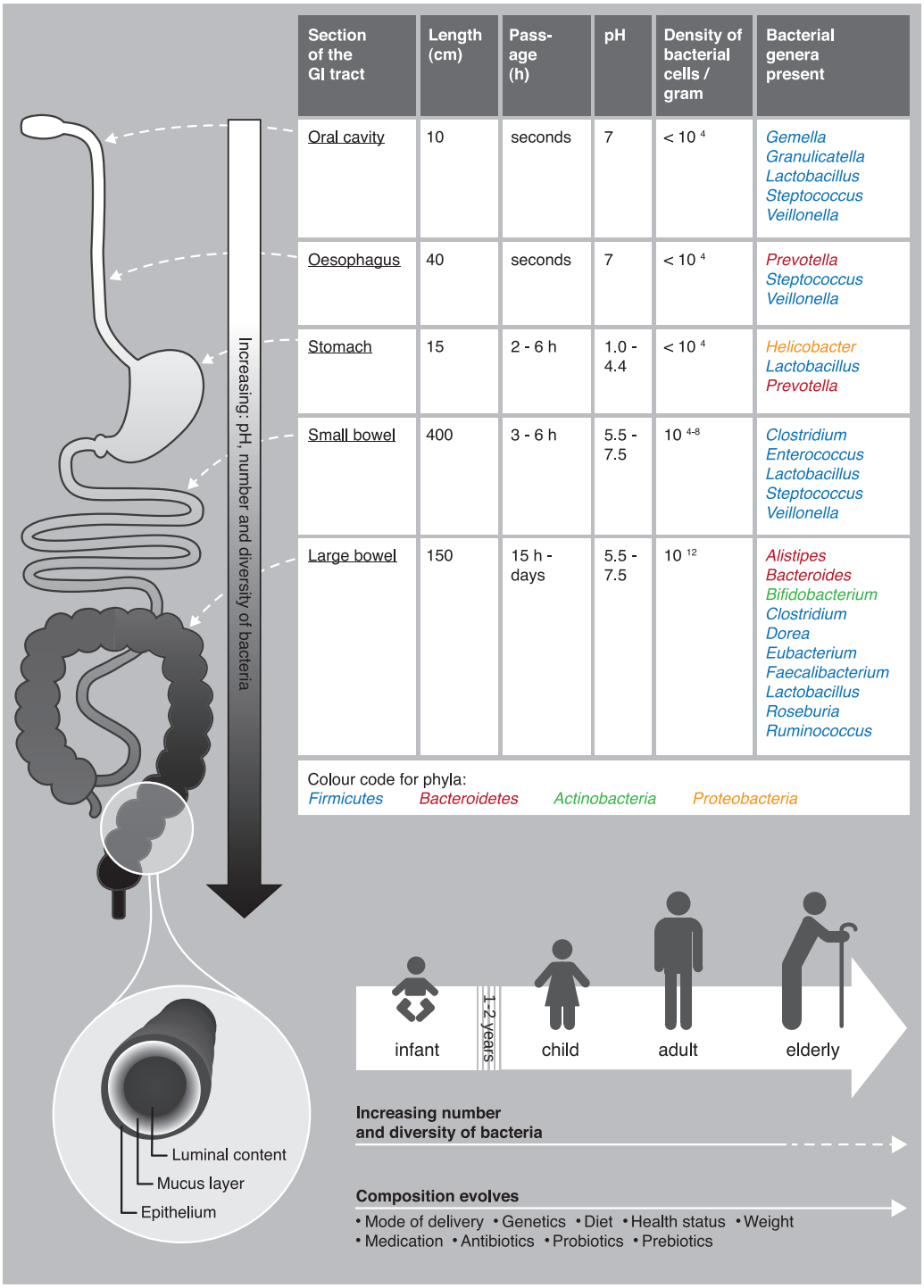
The luminal content and mucosal surface of the human GI tract harbour highly diverse and dense bacterial ecosystems (Eckburg *et al.*, 2005) (Figure 1). Distinct longitudinal variation exists in the composition of the GI microbiota within the 7-m-long GI tract, as the diversity and quantity of bacteria rises along different parts of the intestine towards the rectum, where the density reaches approximately 10^{12} cfu per 1g of faeces. The content of the GI canal undergoes continuous succession, as the bacteria are supplied with nutrients from food and peristalsis moves the colonic content, lubricated by mucus. There is also a flow of liquids and salts, and the pH gradually rises along the intestine from low to neutral (Duncan *et al.*, 2009). The daily load of liquids to the small intestine is approximately nine litres, seven litres of which consist of secretions and two litres comes from the dietary intake (Surawicz, 2010). The small intestine absorbs seven litres of this liquid and the colon absorbs the rest (Surawicz, 2010). The GI tract is highly folded, with crypts and villi within the mucosal surface, adding up to a surface area of 200-400 m^2. Latitudinal variation in the composition of the GI microbiota can be found within each intestinal section, as the luminal and mucosal (Zoetendal

et al., 2002; Kerckhoffs *et al.*, 2010) and the liquid- and particle-associated microbiotas are distinct (Walker *et al.*, 2008). The viscous mucus consists of two layers: a sterile, thinner inner layer and a thicker outer mucus layer occupied by bacteria (Johansson *et al.*, 2008; Johansson *et al.*, 2011).

All three domains of life, *Bacteria*, *Archaea* and *Eukaryotes*, exist in the intestine, as well as many viruses (Rajilić-Stojanović *et al.*, 2007; Reyes *et al.*, 2010). The representation of *Arhaea* is dominated by one species, *Methanobrevibacter smithii* (Eckburg *et al.*, 2005). The predominant bacterial phyla in the GI tract are low G+C Gram-positive *Firmicutes*, high G+C Gram-positive *Actinobacteria* and Gram-negative *Bacteroidetes* and *Proteobacteria* (Figure 1). Other less prevalent phyla include *Verrucomicrobia*, *Fusobacteria* and *Cyanobacteria* (Rajilić-Stojanović *et al.*, 2007). The majority of the bacterial species encountered in the GI tract are strictly anaerobic and have no cultured representatives. Thus, knowledge of their existence is based on sequencing of intestinal community samples (see section 2.2.2). Up to over a thousand bacterial species are estimated to be present in an individual's microbiota (Rajilić-Stojanović *et al.*, 2007; Tap *et al.*, 2009; Qin *et al.*, 2010). In total the bacterial species associated with the human GI tract have been approximated to include from five to tens of thousands of species, depending of the methodology used (Frank *et al.*, 2007; Zoetendal *et al.*, 2008). Some bacteria are transient, some colonise the GI tract temporarily and some are commensals, together forming an equilibrium. It is not clear whether the presence, abundance or proportion of certain bacteria is significant to the host. It has been suggested that a dominant and prevalent set of largely shared microbes, referred to as the common core GI microbiota, is present in the gut of humans. However, the definition of the core GI microbiota is under debate (Rajilić-Stojanović *et al.*, 2009; Tap *et al.*, 2009; Qin *et al.*, 2010; Jalanka-Tuovinen *et al.*, 2011). Very recently, the human gut microbiota has been divided into three main enterotypes dominated by *Bacteroides*, *Prevotella* or *Ruminococcus* (Arumugam *et al.*, 2011).

2.1.3 Role of the GI microbiota

The GI microbiota co-evolves with the host, functioning as an organ within an organ (Camp *et al.*, 2009). The human host is a stable environment supplying nutrients to the microbes, while the microbes have an impact on the host mucosa (Leser & Mølbak, 2009). The gut microbiota forms a functionally uniform ecosystem among human adults (Kurokawa *et al.*, 2007; Turnbaugh *et al.*, 2009; Gosalbes *et al.*, 2011), and the collective microbial genomes in the GI tract encode capabilities lacking from our own genomes (Camp *et al.*, 2009). Dietary carbohydrates are converted to hydrogen, carbon dioxide, methane and short-chain fatty acids (SCFA) (acetate, probionate, butyrate), of which butyrate is the main energy source for gut epithelial cells (Guarner, 2006). The butyrate producers are mainly *Firmicutes*, including members of *Clostridium* clusters XIV and IV (Collins *et al.*, 1994), and are highly abundant in the gut (Louis *et al.*, 2010). The degradation of proteins produces not only SCFAs but also potentially toxic substances. The GI microbiota, however, transforms many potential carcinogens and activates bioactive compounds, produces essential vitamins (such as K, B12 and biotin), contributes to bile acid metabolism and recirculation, affects to the absorption of calcium, magnesium and iron, and participates in the regulation of fat storage (Guarner, 2006; Leser & Mølbak, 2009). Furthermore,



Section of the GI tract	Length (cm)	Pass-age (h)	pH	Density of bacterial cells / gram	Bacterial genera present
Oral cavity	10	seconds	7	$< 10^{4}$	*Gemella* *Granulicatella* *Lactobacillus* *Steptococcus* *Veillonella*
Oesophagus	40	seconds	7	$< 10^{4}$	*Prevotella* *Steptococcus* *Veillonella*
Stomach	15	2 - 6 h	1.0 - 4.4	$< 10^{4}$	*Helicobacter* *Lactobacillus* *Prevotella*
Small bowel	400	3 - 6 h	5.5 - 7.5	10^{4-8}	*Clostridium* *Enterococcus* *Lactobacillus* *Steptococcus* *Veillonella*
Large bowel	150	15 h - days	5.5 - 7.5	10^{12}	*Alistipes* *Bacteroides* *Bifidobacterium* *Clostridium* *Dorea* *Eubacterium* *Faecalibacterium* *Lactobacillus* *Roseburia* *Ruminococcus*

Colour code for phyla:
Firmicutes *Bacteroidetes* *Actinobacteria* *Proteobacteria*

Figure 1. Characteristics of the human GI tract and microbiota (Pei *et al.*, 2004; Aas *et al.*, 2005; Bik *et al.*, 2006; Dethlefsen *et al.*, 2006; Booijink *et al.*, 2007; Tap *et al.*, 2009; Booijink *et al.*, 2010; Sekirov *et al.*, 2010).

the GI microbiota participates in the maturation of the host immune system and in homeostasis (Round & Mazmanian, 2009). The GI microbiota has protective functions, as it acts as a barrier and protects the host from pathogens, a function referred to as colonisation resistance (Stecher & Hardt, 2008).

2.1.4 Health-related disturbances in the GI microbiota

As the GI tract is the body's largest immunological tissue and is exposed to a vast number of bacteria, disturbances in the GI microbiota are expected to be linked to many health problems, as reviewed by Sekirov *et al.* (2010) and Neish *et al.* (2009). The mechanisms underlying such alterations or dysbiosis are still often unknown, and in many cases it remains unresolved whether changes in the GI microbiota are a primary or a secondary phenomenon. Moreover, multifactorial aetiologies affect many health conditions, such as host susceptibility and other environmental factors besides GI microbes. According to the microflora hypothesis (Kirjavainen & Gibson, 1999; Noverr & Huffnagle, 2005), which is an extension to the hygiene hypothesis (Strachan, 1989), the reduced or aberrant microbial exposure results in a cascade of underdeveloped microbiota, immature immunological tolerance and an increased incidence of allergy. Antimicrobial agents not only affect the pathogens against which they are directed, but may also have impact on other members of the intestinal microbiota. Antibiotics reduce the diversity of the gut microbiota and can have long-lasting or even permanent effects on the microbial composition (Jernberg *et al.*, 2007; Dethlefsen *et al.*, 2008; Jakobsson *et al.*, 2010). Acute effects of antibiotic treatment include diarrhoea, whereas long-term consequences are more difficult to perceive. Allergic disease and autism are chronic conditions that have been associated with frequent antibiotic use in childhood (Song *et al.*, 2004; Risnes *et al.*, 2011). Diseases affecting systemic health, inflammatory bowel disease (IBD) and GI tract cancers are among the conditions associated with alterations in the GI microbiota, with some conflicting results (Table 1).

2.2 Molecular methods applied in GI microbial community analysis

2.2.1 The 16S rRNA gene

Culture-independent methods have been widely used in research of the GI microbiota, as approximately of 50-90% of the microbes in the GI tract are currently unculturable (Zoetendal *et al.*, 2008). The methods are often based on detecting the gene coding for the 16S ribosomal ribonucleic acid (16S rRNA gene), which is a component of the 30S subunit of all prokaryotic ribosomes. As the 16S rRNA gene is functionally universal, sufficiently conserved, convenient in size (approximately 1500 bp), and large sequence databases exist for comparison, it is the most often used phylogenetic marker (Woese, 1987). To date, the number of 16S rRNA gene sequences in gene data banks exceeds 1.5 million. The term 'species' requires phenotypic characterisation of a bacterium and is inappropriate when the only available information is the 16S rRNA gene sequence. Therefore, 16S rRNA gene sequences can be grouped according to their similarity in terms of operative taxonomic units (OTUs). A cut-off value of 97-99% similarity between sequences within an OTU is often used to define a species representative, also termed a phylotype. The 16S rRNA gene contains conserved and variable regions that can be applied in detecting and identifying bacteria

Table 1. The microbiota associations in different disease conditions.

Condition	Alteration in GI microbiota compared to healthy	Reference
Systemic disease		
Allergic disease	↓ *Bifidobacterium adolescentis*	Sjögren *et al.* 2009
	↓ *Lactobacillus* spp.	
(disease in childhood)	↓ *Clostridium difficile*	
	↑ *Clostridium* spp.	Kalliomäki *et al.* 2001
	↓ *Bifidobacterium* spp.	
	↑ *Escherichia coli*	Penders *et al.* 2007
	↑ *Clostridium difficile* (alterations in infancy)	
Obesity	↑ *Firmicutes*	Ley *et al.* 2006
	↓ *Bacteroidetes*	Ley *et al.* 2006 and Turnbaugh *et al.* 2009
	↑ *Actinobacteria*	Turnbaugh *et al.* 2009
Type 2 Diabetes	↓ *Firmicutes*	Larsen *et al.* 2010
	↓ class *Clostridia*	
Inflammatory bowel disease		
Ulcerative colitis	↓ *Clostridium* cluster XIV	Sokol *et al.* 2006
	↓ *Firmicutes*	Frank *et al.* 2007
	↓ *Bacteroidetes*	
Crohn's disease	↓ *Clostridium* cluster IV	Sokol *et al.* 2006
	↓ *Firmicutes*	Frank *et al.* 2007
	↓ *Bacteroidetes*	
	↓ *Faecalibacterium prausnitzii*	Sokol *et al.* 2008 and Willing *et al.* 2009
	↑ *Escherichia coli*	Willing *et al.* 2009
	↑ *Enterobacteriaceae*	Willing *et.al.* 2010
	↑ *Ruminococcus gnavus*	
	↓ *Faecalibacterium*	
	↓ *Roseburia*	
GI tract cancers		
Gastric cancer	↑ *Helicobacter pylori*	Correa *et al.* 2007
Colorectal cancer	↑ *Clostridium* spp.	Scanlan *et al.* 2008
	↓ *Collinsella aerofaciens*	Moore & Moore 1995
Other		
Acute appendicitis	↑ *Fusobacterium* spp.	Swidsinski *et al.* 2011
Autistic disorder	↑ *Clostridium* spp.	Song *et al.* 2004 and Parracho *et al.* 2005
Cholelithiasis	↑ *Escherichia coli*	Abeysuriya *et al.* 2008

↑, increase relative to healthy subjects; ↓, decrease relative to healthy subjects.

on different taxonomic levels. However, one factor bringing inaccuracy to the quantification of targeted phylotypes or bacterial groups is the varying copy number of the 16S rRNA gene per microbial genome (Farrelly *et al.*, 1995).

Each step in bacterial community analysis is exposed to biases, starting from cell lysis and DNA extraction (Salonen *et al.*, 2010b). The polymerase chain reaction (PCR) is involved in most of the culture-free assays for microbial community samples that detect the 16S rRNA gene (Table 2). As the amplification is exponential and as the microbial community sample contains DNA from different bacteria in varying ratios, the product always contains a biased quantity of amplicons favouring DNA from the dominant bacterial species. Furthermore, the amplification of high %G+C bacteria is weaker than low %G+C bacteria (von Wintzingerode *et al.*, 1997), even though the %G+C content of the 16S rRNA gene does not vary as much between the species as the genomic %G+C content (Haywood-Farmer & Otto, 2003). The secondary structures formed by the 16S rRNA loops can restrain the polymerase from proceeding in amplification or the sequencing reaction (von Wintzingerode *et al.*, 1997). The formation of chimeras, which are amplicons containing DNA from two or more species, can also occur in the amplification reactions (von Wintzingerode *et al.*, 1997). Furthermore, the primers are prone to annealing with differing efficiency depending on the nucleotide mismatches in the template. Although the conserved domains of the 16S rRNA gene offer primer sites for universal assay applications, no truly universal primers are available that would equally amplify all templates. Nevertheless, despite all the uncertainty caused by the nature of the 16S rRNA gene and technical issues, community analysis can often be considered as quantitative or semiquantitative, depending on the method applied (see section 2.2.1.1).

2.2.1.1 Applicable techniques

The most suitable methodology for GI community analysis depends on the research frame. An overview of the methods is provided in Table 2. The analyses applied in research on the GI microbiota in relation to IBS are presented in Table 3. Profiling the microbial community according to the percentage of genomic guanine and cytosine (%G+C) is an example of a non-quantitative approach (Holben & Harris, 1995). This is often used in combination with other methods (Dicksved *et al.*, 2008; Mäkivuokko *et al.*, 2009). Fingerprinting methods such as denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE) and terminal restriction fragment length polymorphism (T-RFLP) are considered as semiquantitative methods that can be used in monitoring the GI microbiota in general or in focusing on specific groups of bacteria (Vanhoutte *et al.*, 2004; Jernberg *et al.*, 2007; Biasucci *et al.*, 2010). Fluorescent *in situ* hybridisation (FISH) (Mueller *et al.*, 2006) and phylogenetic microarray applications (Palmer *et al.*, 2007; Rajilić-Stojanović *et al.*, 2009; Saulnier *et al.*, 2011) are quantitative nucleotide probe-based methodologies applied to the GI microbiota. Quantitative real-time PCR is a rapid, sensitive and highly quantitative analysis method capable of detecting and quantifying minor representatives in the gut microbiota, and it has therefore been used, for example, in the detection of pathogens and in the monitoring of probiotic interventions (Malinen *et al.*, 2003; Kajander *et al.*, 2007; Rinttilä *et al.*, 2011). Primer- and probe-based methods do not necessarily detect novel phylotypes, since

Table 2. Key methods used to study the intestinal microbial community.

Technique	Description	Features
Culturing	Growing of bacteria in unselective or selective media	• Most of the GI bacteria are unculturable and thus not detected • Provides bacterial isolates
DGGE and TGGE	Separation of the amplified DNA bands in a denaturing or temperature gradient followed by presence, absence and intensity analysis of DNA bands	• Overview of the whole community • Taxonomic level can be adjusted with primer selection • Bands can be isolated for further identification • Reproducible
FISH	Visualisation of intact cells	• Dead and living cells can be separated • Spatial detection of bacteria
%G+C profiling	Genomic community DNA profiling according to the G+C content	• No preliminary information on the taxa is needed
Phylogenetic microarray	Semiquantitative detection of target DNA by hybridisation	• High throughput • Suitable for simultaneously monitoring thousands of microbes in one analysis
qPCR	Detection of the amplified target DNA	• Highly sensitive • Quantitative • High throughput
Sequencing	Provision of nucleotide-level information on the sequenced amplicons	• Enables novel sequence detection • Phylogenetic analysis possible • Suitable for simultaneously monitoring thousands of microbes in one analysis
T-RLFP	Restriction digestion of amplicons providing a pattern of the terminal labelled fragments	• Overview of the whole community • Fast

DGGE, denaturing gradient gel electrophoresis; TGGE, temperature gradient gel electrophoresis; FISH, fluorescent *in situ* hybridisation; %G+C, percentage guanine plus cytosine; qPCR, quantitative real-time polymerase chain reaction; T-RFLP, terminal-restriction fragment length polymorphism.

the design of primers and probes is dependent on the available sequence data. Methods based on sequencing are ideal for mapping the diversity of the faecal microbiota and they supply sequence information for oligonucleotide and probe design.

Metagenomic approaches examine all genetic content in a community to observe the genetic potential and in addition provide phylogenetic information through the analysis of 16S rRNA genes (Gill *et al.*, 2006; Manichanh *et al.*, 2006; Kurokawa *et al.*, 2007; Qin *et al.*, 2010). Whole genome data-bases presently cover 396 resident GI tract bacteria (September 2011, http://www.hmpdacc-resources.org/cgi-bin/img_hmp/main.cgi; Markowitz *et al.*, 2010), which can be used to annotate metagenomic sequence data. Microbial community analysis provides information on the microbes present in certain health conditions, but does not indicate whether the microbes are a result or a cause of the condition. Functions and host-microbe interactions can be investigated using bacterial isolates, animal models, *in vitro* models and different -omics approaches, including proteomics, transcriptomics, metabolomics (Zoetendal *et al.*, 2008; Hattori & Taylor, 2009) and meta-transcriptomics (Gosalbes *et al.*, 2011).

2.2.2 Sequencing of the GI microbiota

The development of sequencing technologies and the application of microbial ecology statistics combined with computational tools have revolutionised the culture-independent approaches to microbial communities, including the GI microbiota (Figure 2). Studies on the characterisation of the normal healthy faecal microbiota with Sanger dideoxy sequencing of the 16S rRNA gene clone libraries started in the 1990´s and resulted in a few hundred sequences from a few individuals (Wilson & Blitchington, 1996). The estimated numbers of faecal bacterial phylotypes in individuals in these studies were some hundreds. The first large-scale 16S rRNA gene study on the faecal microbiota based on Sanger dideoxy sequencing was published by Eckburg *et al.* (2005), and since then estimates have been around a thousand phylotypes in an individuals gut. Second-generation pyrosequencing methods made sequencing more effective and economical and changed the proportions of phyla (Andersson *et al.*, 2008).

The sequencing studies performed thus far to characterise the healthy human GI microbiota have analysed a combined total of only some hundreds of individuals representing a restricted range of nationalities (Figure 2). Furthermore, the comparison of results from different studies is problematic due to the considerable variation in methodological parameters, such as sample preparation, DNA extraction, primer and probe selection and the region of the 16S rRNA gene sequenced (Salonen *et al.*, 2010b). The phylum-level deviation between these studies has also been broad. The development of third-generation sequencing techniques will facilitate even more extensive sequencing to explore the microbiota of greater number of individuals with better coverage (Eid *et al.*, 2009).

2.2.2.1 Microbial community structure analysis

In culture-free microbial community analysis, a high sequence similarity in the 16S rRNA gene sequence forms a species-like concept referred to as phylotype or OTU. Statistical methods to assess the diversity and structure of the microbiota have been adapted from macroorganisms, and conclusions should be drawn considering the limitations introduced

due to the prokaryotic nature of the sequence data. Several web applications have facilitated convenient analysis for affiliating phylotypes and observing the phylogenetic structure of microbial communities (Schloss & Handelsman, 2005; Schloss & Handelsman, 2006; Lozupone & Knight, 2008; Cole *et al.*, 2009).

The coverage of sampling can be tested to monitor the depth of community sequencing. This can be calculated with Good's formula [1-(n/N)] (Good, 1953), in which 'N' is the number of cloned sequences and 'n' represents the number of single-clone OTUs. The cumulative number of phylotypes versus sequenced clones can be plotted as an accumulation curve. A curve that reaches the plateau indicates a well sampled community (Hughes *et al.*, 2001). In addition, rank-abundance curves can be drawn to visualise the community structure, in which the abundance of each phylotype is plotted against the species ordered from the most abundant to the least abundant (Hughes *et al.*, 2001).

Diversity consists of species richness and evenness. High diversity is generally thought to be beneficial for an ecosystem due to its balancing effect on the structure of a community under stressful conditions. Diversity is often used as a term of richness, referring to the number of phylotypes in an ecosystem. Species evenness refers to the relative abundance or proportion of individuals among the species. Estimators such as Chao and the abundance-based coverage estimator (ACE) can be used to estimate the true richness (Chao, 1984; Chao & Lee, 1992). Diversity, on the other hand, can be indicated with indices that combine the dimensions of richness and evenness, such as Simpson's and Shannon's indices for diversity (Shannon, 1948; Simpson, 1949).

2.3 Irritable bowel syndrome (IBS)

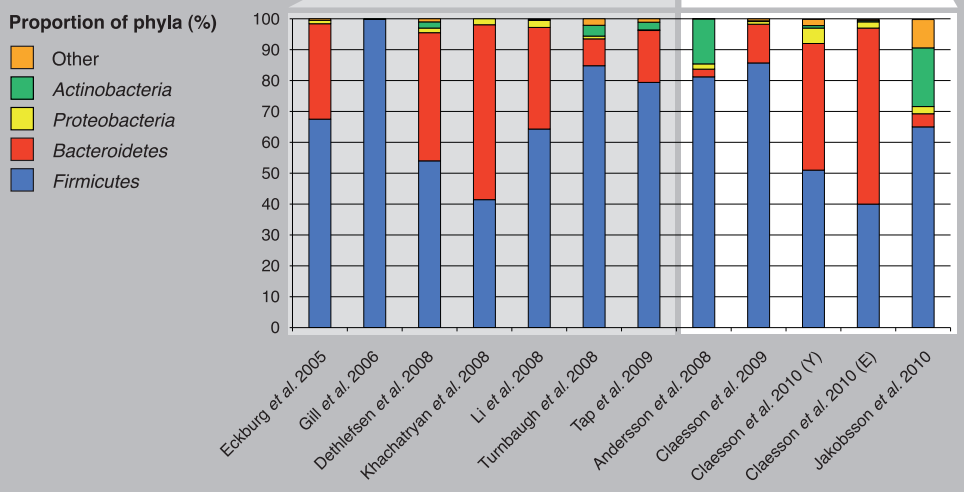
2.3.1 Definition of the disorder

Irritable bowel syndrome (IBS) is a functional bowel disorders (Longstreth *et al.*, 2006). The main symptom is abdominal pain or discomfort occurring at sufficient frequency in association with defecation-related alterations (Longstreth *et al.*, 2006). The syndrome affects 10-20% of the Western adults and adolescents and has a female predominance (Longstreth *et al.*, 2006; Talley, 2008). The prevalence in Finland is 5.1-16.2%, depending on the criteria used (Hillilä & Färkkilä, 2004). The aetiology of IBS is unclear, but several biological, physiological and psychosocial factors are considered to be involved in the emergence of this heterogeneous disorder (Öhman & Simrén, 2010). Furthermore, genetics may play a role in the syndrome, as shown in a twin study (Bengtson *et al.*, 2006). Patients have many somatic (gastrointestinal conditions, headache, backache) and psychiatric

Figure 2. Selected studies of faecal microbiota of healthy subjects based on 16S rRNA gene sequencing.
The phyla were determined by searching for the sequences using the ribosomal database (RDB) classifier (Cole *et al.*, 2009) or adapted from publications (Andersson *et al.*, 2008; Khachatryan *et al.*, 2008; Claesson *et al.*, 2010; Jakobsson *et al.*, 2010). Two subjects were excluded due to a diagnosis of UC and the consumption of antibiotics (Claesson *et al.* 2009); Obese subjects were excluded (Turnbaugh *et al.* 2009); Three normal-weighed subjects were included (Zhang *et al.* 2009); Y, younger subjects; E, elderly subjects.

Sequencing studies on healthy human GI microbiota

	Conventional Sanger dideoxy sequencing of clone libraries		Pyrosequencing
	Low throughput	**High throughput**	
Sequences per study	10^{1} - 10^{2}	10^{3} - 10^{4}	10^{4} - $10^{5(6)}$
Highest estimated amount of phylotypes	> 500	> 1 000	> 5 000
Healthy subjects (per study)			
Number of subject sampled	1 - 3	2 - 154	3 - 154
Age range	27 - 60	1.5 - 95	22 - 87
Country where subject recruited	GB, Japan, Spain, France	Armenia, China, France, The Netherlands, USA	Ireland, Sweden, USA
Publications	Wilson *et al.* 1996 Suau *et al.* 1999 Bonnett *et al.* 2002 Hayashi *et al.* 2002 Delgado *et al.* 2006	Eckburg *et al.* 2005 Gill *et al.* 2006 Dethlefsen *et al.* 2008 Khachatryan *et al.* 2008 Li *et al.* 2008 Turnbaugh *et al.* 2008 Tap *et al.* 2009	Andersson *et al.* 2008 Dethlefsen *et al.* 2008 Turnbaugh *et al.* 2008 Zhang *et al.* 2008 Claesson *et al.* 2009 Claesson *et al.* 2010 Jakobsson *et al.* 2010



(anxiety, depression, insomnia) comorbidities (Hillilä *et al.*, 2007). IBS sufferers are not predisposed to severe illness, but the intestinal disorder significantly diminishes their QOL (Si *et al.*, 2004; Tana *et al.*, 2010). Moreover, IBS has an economic impact on the society via work absenteeism and increased medical costs (Hillilä *et al.*, 2010).

2.3.1.1 Diagnostic criteria

The diagnosis of IBS is based on symptoms, as no biological markers or distinct physiological characteristics are available for the syndrome (Clarke *et al.*, 2009). The criteria for IBS have frequently been refined and have so far included the Manning (Manning *et al.*, 1978), Rome I (Thompson *et al.*, 1992), Rome II (Thompson *et al.*, 1999) and most recently the Rome III (Longstreth *et al.*, 2006) criteria. Most of the recently published studies have enrolled subjects by applying the Rome II criteria. The Rome III criteria are as follows: "*Recurrent abdominal pain or discomfort should be observed for at least three days every month, for three months consecutively. The pain or discomfort has to have two of the following three features: Improvement with defecation, onset associated with a change in frequency of stool or form (appearance) of stool. Furthermore, the criterion should be fulfilled for the last three months with symptom onset at least six months prior to diagnosis*" (Longstreth *et al.*, 2006). Based on the predominant stool habit (Lewis & Heaton, 1997), IBS sufferers are further classified into the following categories: diarrhoea-predominant IBS (IBS-D), constipation-predominant IBS (IBS-C) and mixed symptom subtype IBS (IBS-M). In addition, patients whose bowel habit symptoms change over time are referred to as alternating symptom subtype IBS (IBS-A) patients. Subjects who cannot be categorised into these groups are referred to as unclassified IBS (Longstreth *et al.*, 2006). The main modifications of the criteria in Rome III compared to the previous versions relate to the timely demands of symptom duration, i.e. the occurrence of symptoms for a period before the diagnosis. Symptoms such as mucus in the stool, bloating, straining, urgency and the sensation of incomplete evacuation are no longer included in the symptom criteria, but are listed as supportive symptoms. Alarm signs not typical for IBS include onset of symptoms at 50 years or older, unintentional weigt loss, nocturnal diarrhoea, anemia, blood in faeces, and familial history of colon cancer, celiac disease or IBD (Khan & Chang, 2010). In Finland, patients are tested for lactose intolerance and celiac disease before IBS diagnosis as the symptoms may resemble IBS symptoms (Hillilä, 2009).

2.3.1.2 Treatment

Presently, there is no cure for IBS, but the symptoms can be alleviated; Diarrhoea can be treated with antidiarrhoeals like loperamide, constipation with fibers and abdominal pain with antidepressants (Khan & Chang, 2010). The drugs alleviate symptoms for only a part of the patients. As the syndrome itself does not predispose to severe illness, there is a demand for safe treatments without side-effects. Diet changes and avoidance of sressfull circumstances alleviate the symptoms for most of the patients. Furthermore, probiotics have shown to alleviate the symptom of IBS as discussed later in Section 2.4.

2.3.2 Pathophysiology

Suggested pathophysiological factors in IBS include visceral hypersensitivity, immune activation, abnormal gut motility, secretion and permeability, dysfunction in the brain-gut axis and altered gut microbiota (Öhman & Simrén,

2007; Parkes *et al.*, 2008; Öhman & Simrén, 2010). The distorted interaction between these factors contributes to IBS symptoms, which are exacerbated by stress (Khan & Chang, 2010). Low-grade colonic mucosal inflammation associated with IBS may play a role in the pathogenesis, as IBS has similar symptoms to UC in remission and celiac disease (Öhman & Simrén, 2010). Serine protease activity suggested to originate from a perturbed GI microbiota in the colon may be a pathophysiological factor in the development of IBS-D (Róka *et al.*, 2007). Furthermore, faecal supernatant from IBS-D caused visceral hypersensitivity and increased paracellular permeability *in vitro*, with a mechanism that was mediated by protease-activated receptor two (PAR-2) (Gecse *et al.*, 2008). Recently, Brint *et al.* (2011) found an altered distribution of toll-like receptors (TLR) in IBS, indicating a role for interactions between the GI microbiota and the innate immune system of the host in the pathogenesis of IBS.

2.3.2.1 Post-infectious IBS

Some patients fulfil the diagnostic criteria for IBS after a gastrointestinal infection, when it is referred to as post-infectious IBS (PI-IBS). Enteric infections caused by bacteria (*Campylobacter*, *Escherichia coli* 0157:H7, *Salmonella*, *Shigella*), noroviruses, parasites (*Giardia duodenalis*, *Trichinella britovi*) or travellers' diarrhoea due to unspecified infectious agents have been shown to lead to IBS in 3.7-36% of patients (Rodríguez & Ruigómez, 1999; Spiller & Garsed, 2009). The predominant bowel disturbance in PI-IBS is diarrhoea. The prognosis for PI-IBS patients is better than for IBS patients in general, but the symptoms can manifest for years. A severe multi-pathogen outbreak caused by *E.coli* O157:H7 and *C. jejuni* infected over 2300 people in Walkerton (Ontario, Canada) and served as a valuable cohort for studying PI-IBS, as 36.2% of the patients developed IBS after 2-3 years of infection (Marshall *et al.*, 2006). Host genes playing important roles in bacterial recognition, inflammatory responses and epithelial integrity were associated with the development of PI-IBS in the same cohort as determined by Villani *et al.* (2010). Antibodies against certain bacterial flagellins have also been more frequently found in patients with IBS, especially PI-IBS (Schoepfer *et al.*, 2008).

2.3.2.2 Small intestinal bacterial overgrowth (SIBO)

Antibiotics have been used as therapeutic agents in IBS in the context of small intestinal bacterial overgrowth (SIBO) (Parkes *et al.*, 2008), a condition where bacterial counts in the small intestine are elevated. SIBO is suggested to be a marker in IBS (Pimentel *et al.*, 2000; Pimentel *et al.*, 2003). The connection between SIBO and IBS is unclear and criticism has been directed at the lactulose/glucose hydrogen breath test (L/GHBT) used to diagnose SIBO, which has not been validated and is unable to properly discriminate between healthy and IBS subjects (Posserud *et al.*, 2007; Bratten *et al.*, 2008). Furthermore, treating IBS patients with antibiotics to reduce excessive bacterial counts has been suggested to suppress the growth of bacteria in the colon, rather than in the small intestine (Yu *et al.*, 2010). Antibiotics may actually provoke functional abdominal symptoms and even trigger IBS (Maxwell *et al.*, 2002). Thus, regular consumption of antibiotics is not recommended.

2.3.2.3 Colonic fermentation

Abnormal colonic fermentation, associated with alterations in the activity of hydrogen-

consuming bacteria, has been linked with IBS in a study by King *et al.* (1998), in which symptoms in the subjects improved simultaneously with a fall in hydrogen and methane excretion due to an exclusion diet. More recently, altered metabolism in protein and carbohydrate fermentation has been linked to the microbiota in IBS as indicated by Ponnusamy *et al.* (2011) and Tana *et al.* (2010). As common IBS symptoms include bloating and flatulence (Hungin *et al.*, 2003), there is thus suggested to be a link between altered colonic fermentation and gas in IBS (Parkes *et al.*, 2008). The gaseous bacterial fermentation products of undigested carbohydrates in the gut microbiota include short-chain fatty acids, carbon dioxide, methane and hydrogen (Parkes *et al.*, 2008). Moreover, impaired handling rather than the load of gas in IBS patients has also been suggested trigger symptoms (Salvioli *et al.*, 2005).

2.3.2.4 Alterations of the microbiota

The overall faecal microbiota in IBS has been shown with various methods to be different from that of healthy individuals (Malinen *et al.*, 2005; Kassinen *et al.*, 2007; Codling *et al.*, 2009; Noor *et al.*, 2010; Ponnusamy *et al.*, 2011; Rajilić-Stojanović *et al.*, 2011). The IBS-associated GI microbiota has also been shown to be more unstable than that in healthy subjects (Maukonen *et al.*, 2006b). In particular, the faecal microbiota of IBS-D patients is the most distinct, whereas IBS-C appears to be the most similar to that of control subjects devoid of gastrointestinal symptoms (Kassinen *et al.*, 2007; Lyra *et al.*, 2009). Moreover, the diversity and inter-individual variation of IBS related faecal microbiota has also diverged from that of healthy (Codling *et al.*, 2009; Noor *et al.*, 2010; Carroll *et al.*, 2011; Ponnusamy *et al.*, 2011). Alterations in the microbiota have been seen in all of the main bacterial phyla present in the GI tract, i.e. *Firmicutes*, *Bacteroidetes*, *Proteobacteria* and *Actinobacteria* (Table 3). Similarly to healthy subjects, nearly half of IBS subjects harbour methanogen producers (*Archaea*) (Scanlan *et al.*, 2008a). The IBS subtype-specific features observed in culture-independent studies have been recently reviewed by Salonen *et al.* (2010). The authors stated that the inconsistency of the outcomes in the studies of IBS microbiota reflects a loss of homeostasis in the GI tract of IBS patients, as a high or low degree of variation is a sign of ongoing succession or outgrowth of certain taxa in a community, respectively. Furthermore, intestinal ecological dysbiosis was suggested as a pathophysiological factor in IBS, meaning that the syndrome is caused by the status of community-level multispecies alterations (Salonen *et al.*, 2010a).

Actinobacteria

Alterations in *Bifidobacterium* spp. from the phylum *Actinobacteria* have most often been reported in comparative studies on IBS-related microbiota (Table 3). IBS patients have been determined to have reduced quantities of total bifidobacteria in their faeces by culturing (Balsari *et al.*, 1982; Si *et al.*, 2004; Enck *et al.*, 2009b) and with molecular methods (Kerckhoffs *et al.*, 2009; Ponnusamy *et al.*, 2011; Rajilić-Stojanović *et al.*, 2011). More specifically, decreased levels of *Bifidobacterium catenulatum* have been demonstrated in IBS in both faeces (Malinen *et al.*, 2005; Kerckhoffs *et al.*, 2009) and mucus (Kerckhoffs *et al.*, 2009). IBS-D and IBS-C form the extremities for the counts of *Bifidobacteria* spp. and the *Collinsella aerofaciens* phylotype, as IBS-D had reduced and IBS-C elevated levels (Kassinen *et al.*, 2007; Lyra *et al.*, 2009).

Table 3. Comparative studies of the GI microbiota in IBS patients and healthy subjects based on culturing and molecular methods applying the 16S rRNA gene.

Main finding (IBS vs. healthy)	Method	Number of HC/IBS subjects (criteria)[1]	Study
↓ *Bacteroidetes* ↑ *Firmicutes* ↓ *Bifidobacterium* spp. ↓ *Methanobrevibacter* spp.	Microarray qPCR	62/46 (Rome II, S)	Rajilić-Stojanović *et al.* 2011
Lower biodiversity (F)	T-RFLP	21/16 (Rome III, IBS-D) faecal+ colonic mucosal biopsy	Carroll *et al.* 2011
↑ phylogenetical diversity ↑ *Gammaproteobacteria* ↑ *Ruminococcus*-like phylotype ↑ *Veillonella* spp. ↓ *Bacteroidetes* spp.	16S rRNA gene pyrosequencing Microarray	22/22 (Rome III, children, S)	Saulnier *et al.* 2011
↑ *Staphylococcus aureus*	qPCR	23/96 (Rome I and II, S)	Rinttilä *et al.* 2011[3]
↑ total bacteria ↑ *Bacteroidetes* ↑ *Lactobacillus* spp. ↓ *Bifidobacterium* spp. ↓ *Clostridium coccoides* -group	DGGE qPCR	8/11 (Rome II, S)	Ponnusamy *et al.* 2011[4]
↑ number of *Lactobacillus* spp. (F) ↓ aerobic bacteria (F)	qPCR Culturing	10/10 (Rome III, IBS-D) faecal+ colonic mucosal biopsy	Carroll *et al.* 2010
↑ biological variability ↓ biodiversity ↓ *Bacteroides vulgatus, B. ovatus, B. uniformis, Parabacteroides* spp.	DGGE	22/11 (Rome II, U)	Noor *et al.* 2010
↑ *Pseudomonas aeruginosa*	DGGE qPCR	20/37 (Rome II, S) faecal+ duodenal mucosal brush	Kerckhoffs *et al.* 2010
↑ *Veillonella* spp. ↑ *Lactobacillus* spp.	qPCR Culturing	26/26 (Rome II, S)	Tana *et al.* 2010[2,4]
↑ *Ruminococcus torques* 94% (IBS-D) ↓ *Clostridium thermosuccinogenes* 85% (IBS-D) ↑ *Ruminococcus bromii* (IBS-C) ↓ *Ruminococcus torques* 93% (IBS-M)	qPCR	15/20 (Rome II, S)	Lyra *et al.* 2010[3]
↓ variation	DGGE	33/ 47 (Rome II, U) faecal+ 9 IBS: colonic mucosal biopsy	Codling *et al.* 2009
↓ *Bifidobacterium* spp.	Culturing	7765 (ND)	Enck *et al.* 2009

↓ *Bifidobacterum* spp. (F) ↓ *Bifidobacterium catenulatum* (F+M)	FISH	26/41 (Rome II, S) faecal+ duodenal brush	Kerckhoffs *et al.* 2009
↓ *Collinsella aerofaciens* ↓ *Coprococcus eutactus* 97% ↓ *Clostridium cocleatum* 88% (↓) *Bifidobacterium catenulatum / pseudocatenulatum* (↑) *Ruminococcus torques* 94%	16S rRNA gene cloning and Sanger sequencing of the %G+C fractions qPCR	23/24 (Rome II, S)	Kassinen *et al.* 2007[3]
↑ instability ↓ *Clostridium coccoides -Eubacterium rectale* group (IBS-C)	DGGE TRAC	16/16 (Rome II, S)	Maukonen *et al.* 2006[3]
↓ *Clostridium coccoides* group ↓ *Bifidobacterium catenulatum* ↑ *Veillonella* spp. (IBS-C) ↓ *Lactobacillus* spp. (IBS-D) ↓ *Bifidobacterium* spp. (IBS-D)	qPCR	22/27 (Rome II, S)	Malinen *et al.* 2005[3]
↑ coliforms ↑ aerobe:anaerobe ratio	Culturing PCR-DGGE	25/26 (Rome II, S)	Mättö *et al.* 2005[3]
↑ *Eubacterium rectale - Clostridium coccoides* -group	FISH	20/20 (Rome II, U) Ileum and colon mucosal biopsy	Swidsinski *et al.* 2005
↓ *Bifidobacterium* spp. ↑ *Enterobacteriaceae*	Culturing	25/25 (Rome II, U)	Si *et al.* 2004[2]
↓ coliforms ↓ *Lactobacillus* spp. ↓ *Bifidobacterium* spp.	Culturing	20/20 (Manning, U)	Balsari *et al.* 1982

F, finding from faecal samples; M, finding from mucosal samples; S, subtyped IBS patients; U, unsubtyped IBS patients; HC, healthy controls; qPCR, quantitative real-time polymerase chain reaction; DGGE, denaturing gradient gel electrophoresis; FISH, fluorescent *in situ* hybridisation; TRAC, transcript analysis with the aid of affinity capture; TRFLP, terminal restriction fragment length polymorphism; 1) Faecal samples if not otherwise mentioned; 2) quality of life (QOL) monitored; 3) Samples or fraction of the samples from the same patient cohort; 4) Metabolites monitored.

Gram-negative bacteria

Noor *et al.* (2010) found *Bacteroides* spp. less frequently and in lower quantities in the faeces of IBS subjects than in controls. Recently, the number of *Bacteroides* spp. was also decreased in IBS in a study by Rajilić-Stojanović *et al.* (2011), but elevated in a study by Ponnusamy *et al.* (2011). Another predominant Gram-negative phylum in the intestine, *Proteobacteria*, has shown alterations in association with IBS: *Enterobacteriaceae* have been elevated (Si *et al.*, 2004; Mättö *et al.*, 2005) or decreased (Balsari *et al.*, 1982) in IBS. Recently, *Gammaproteobacteria* was found to be increased in pediatric patients (Saulnier *et al.*, 2011). With more specific assays of *Protebacteria*, *Pseudomonas aeruginosa* was recently shown to be more frequently detected at elevated levels in IBS (Kerckhoffs *et al.*, 2010).

Firmicutes

Within the phylum of *Firmicutes*, *Lactobacillus* spp. are reported to be increased in unsubtyped IBS (Tana *et al.*, 2010; Ponnusamy *et al.*, 2011) and in IBS-D (Carroll *et al.*, 2010). Conversely, an early study by Balsari *et al.* (1982) reported reduced quantities of lactobacilli in IBS, as did Malinen *et al.* (2005), in which the reduction was seen in IBS-D. Recently, 17% of IBS patients were found positive for *Staphylococcus aureus*, whereas none of the healthy controls were positive (Rinttilä *et al.*, 2011). Furthermore, increased *Veillonella* spp. has been detected among IBS-C (Malinen *et al.*, 2005) and unsubtyped IBS subjects (Tana *et al.*, 2010). In the latter study (Tana *et al.*, 2010), besides higher counts of *Veillonella* spp. and *Lactobacillus* spp., the levels of acetic acid, propionic acid and total organic acids in faeces were also higher in the IBS patient samples. In addition, patients with high acetic acid or propionic acid levels had significantly worse GI symptoms and QOL. Interestingly, *Veillonella* spp. and *Lactobacillus* spp. are producers of acetic and propionic acid. Treem *et al.* (1996) measured faecal fatty acid concentrations at baseline and after incubation *in vitro* with different carbohydrates. Subjects with IBS-D had a consistently different pattern of SCFA production than controls seen as decreased total SCFAs, a lower percentage of acetate, and a higher proportion of n-butyrate (Treem *et al.*, 1996). Furthermore, the abundance of *Lactobacillus* spp. and *Clostridium* spp. in IBS was recently associated with high levels of amino acids and phenolic compounds (Ponnusamy *et al.*, 2011).

Studies on the most abundant bacterial group in the gut, *Clostridium coccoides* (also known as *Clostridium* cluster XIV within *Firmicutes*), have reported fewer bacteria of this group in IBS patients than in controls (Malinen *et al.*, 2005; Ponnusamy *et al.*, 2011). Contrary to this, Maukonen *et al.* (2006) observed such reduction only in the IBS-C group from the same sample cohort as used by Malinen *et al.* (2005), most probably due to the different methodologies used. On the mucosa in some IBS patients, the abundance of *Clostridium* XIV group has exceeded 90% of the total microbes (Swidsinski *et al.*, 2005). Phylotypes of this group have been studied in more detail by Kassinen *et al.* (2007) and Lyra *et al.* (2009). The most apparent result is that a phylotype with a 16S rRNA gene sequence similarity of 94% to a mucus-degrading *Clostridium* XIV bacterium *Ruminococcus torques*, is abundant in IBS-D (Lyra *et al.*, 2009).

2.4 Probiotics in IBS

2.4.1 Characteristics of probiotics

According to the current definition by FAO/WHO, probiotics are: "Live microorganisms which when administered in adequate amounts confer a health benefit on the host" (WHO, 2002). Strains of *Lactobacillus* spp. and *Bifidobacteria* spp., as well as *Propionibacterium* spp., *Enterococcus* spp. and *Echerichia coli* and the yeast, *Saccharomyces boulardii*, have most often been used as probiotics. Probiotics should be safe to the consumer and they should also have proven beneficial effects on the host. The beneficial effects seen are strain-specific, and thus the results obtained cannot be extrapolated to other strains. Moreover, each health condition has to be evaluated independently (Rijkers *et al.*, 2010). Probiotics are added to food products or consumed as capsules at a typical daily dose of approximately 10^8-10^{10} cfu. The cells should remain viable during technological processing, throughout the shelf life of the product they are added to, and while passing the GI

tract (Rijkers *et al.*, 2010). In addition, prebiotics are often used with probiotics to gain a synergistic effect (synbiotics). Prebiotics are indigestible food compounds that selectively promote the growth or activity of certain bacteria (Gibson & Roberfroid, 1995).

2.4.2 Health effects in the gut

Probiotics have long been believed to be beneficial in maintaining enteric homeostasis and preventing disease. The first observations of beneficial lactic acid bacteria were made by Metchnikoff at the beginning of the 20th century. Probiotics can act in the gut lumen by fortifying the colonisation resistance. They may enhance the gut barrier by producing antimicrobial agents such as bacteriocins and SCFAs that kill pathogens or inhibit their growth. Probiotics also compete with commensals and pathogens in binding to the mucosa and epithelial cells. Furthermore, certain probiotics have an effect on epithelial cells, in which they promote mucus secretion and affect gut permeability via epithelial cell tight junctions. They may also have an immunomodulative effects through both the innate and adaptive arm of the immune system. (Delcenserie *et al.*, 2008; Ohland & Macnaughton, 2010).

Specific probiotic strains have been demonstrated to be efficient in the treatment of various GI diseases and conditions, including IBS (Table 4). Probiotics have also been found to prevent and reduce the duration of diarrhoea caused by rotavirus, *Clostridium difficile*, travelling and antibiotics (Wolvers *et al.*, 2010). Furthermore, probiotics have been shown to help in the eradication of *Helicobacter pylori* and in preventing necrotising enterocolitis (NEC) in infants (Wolvers *et al.*, 2010). Probiotics are effective in the mainteinance of remission in UC and pouchitis, but interestingly are less efficient in Crohn's disease (CD) (Haller *et al.*, 2010).

2.4.3 Clinical trials of probiotics in IBS

Studying the effects of probiotics in IBS is justified by several reasons. The current medical treatments for IBS patients are low in efficiency and many predispose to many side effects (Khan & Chang, 2010), and there is thus a need for better and safer treatments, the criteria which probiotics might meet. The GI microbiota in IBS has also proven to be aberrant in comparison to healthy subjets, as reviewed in section 2.3.2.4. Furthermore, probiotics are suggested to be able to modulate gut transit, visceral hypersensitivity, intestinal gas production and inflammatory responses (Haller *et al.*, 2010), which are all important factors in IBS (see section 2.3.2).

The symptoms of IBS have been monitored in many large randomised placebo-controlled trials with different probiotic strains, including studies using multispecies probiotic mixtures (Table 4). *L. rhamnosus* GG has been the most frequently used probiotic strain as it has been used in six out of 27 trials. The potential mechanism of action associated with alleviation of symptoms of IBS has been reported in some of the studies. Delay in colonic transit was shown in IBS-D patients receiving VSL#3 (*Bifidobacterium longum*, *B. infantis*, *B. breve*, *Lactobacillus acidophilus*, *L. casei*, *L. deldrueckii* subsp. *bulgaricus*, *L. plantarum*, *Streptococcus salivarius* subsp. *thermophilus*) (Kim *et al.*, 2005). In another study with a probiotic mixture with *L. rhamnosus* GG, *L. rhamnosus* Lc705, *Propionibacterium freudenreichii* ssp. *shermanii* JS and *B. animalis* ssp. *lactis* Bb12 the supplement appeared to stabilise the microbiota composition (Kajander *et al.*, 2008). In a study with a single

strain of *B. infantis* 35624 the interleukin (IL)-10/IL-12 ratio, which has been shown to be low in IBS, was normalised (O'Mahony *et al.*, 2005). More recently, *L. rhamnosus* GG normalised the increased intestinal permeability of children with IBS (Francavilla *et al.*, 2010).

The effects of probiotic interventions on IBS are difficult to compare due to differences in probiotic strains and doses used, the duration of the studies, small number of subjects and overall study designs (McFarland & Dublin, 2008; Spiller, 2008; Brenner *et al.*, 2009; Hoveyda *et al.*, 2009; Moayyedi *et al.*, 2010; Parkes *et al.*, 2010) (Table 4). A high placebo response has also been characteristic in these studies. With caution it may be concluded, that both single and multiple strain probiotic supplements of various strain types have more often been effective than uneffective (in 22 out of 27 studies). The effect of the probiotic theraphy has been more pronounced on the GI symptoms than on the quality of life (positive effect of QOL in six out of 12 studies) (Table 4).

Table 4. Selected randomised, double-blind, placebo-controlled trials in IBS with different probiotic strains carried out since 2000 and their clinical outcome regarding GI symptoms, bowel habit and QOL.

Study	Strains, daily dose (cfu), product	Number of patients, age range (mean), criteria for IBS	Duration of the treatment	Clinical outcome regarding GI symptoms and bowel habit	Effect on QOL
Studies on single probiotic strains					
Guyonnet *et al.* 2007	*Bifidobacterium animalis* DN-173010, 1,25 x 10^{10} (+ *Streptococcus thermophilus*, *Lactobacillus bulgaricus* 1,2 x 10^{9}), yogurt	267 20-65 (49) Rome II (IBS-C)	6 weeks	A beneficial effect on bloating. Increase in defecation frequency in subjects with less than 3 times per week	+
Guglielmetti *et al.* 2011	*B. bifidum* MIMBb75, 1 x 10^{9}, capsule	122 18-68 Rome III	4 weeks	Reduction in the global assessment of IBS symptoms, Improved in pain/discomfort, distension/bloating, urgency and digestive disorder	+
Whorwell *et al.* 2006	*B. infantis* 35624, 1 x 10^{6}/1 x 10^{8}/ 1 x 10^{10}, capsule	362 females 18-65 Rome II	4 weeks	Dose of 1 x 10^{8} : Alleviation of abdominal pain, symptom score, bloating, bowel dysfunction, incomplete evacuation, straining and flatulence	-
Agrawal *et al.* 2008	*B. lactis* DN173010, 1.25 x 10^{10}, (+ *S. thermophilus*, *L. bulgaricus*, 1.2 x 10^{9}), fermented milk or non-fermented dairy product	34 females 20-69 (40) Rome III (IBS-C)	4 weeks	Reduction in abdominal distension and bloating , acceleration of bowel transit	ND
Enck *et al.* 2009	*Escherichia coli* DSM17252, 1.1-6.8 x 10^{7}, oral liquid	298 18-76 (50) WONCA[2]	8 weeks	Improvement in general symptom and abdominal pain scores	ND
Sen *et al.* 2002[1]	*L. plantarum* 299V, 6.3 x 10^{9}, rose-hip drink	12 23-61 (41) Rome I or II	4+4 weeks cross-over	No effect	ND
Niedzielin *et al.* 2001	*L. plantarum* 299V, 2 x 10^{10}, fruit drink	40 27-63 (45) Manning (adjusted)	4 weeks	Reduction in abdominal pain. A trend towards normalization of defecation frequency in IBS-C	ND

Nobaek *et al.* 2000[1]	*L. plantarum* DSM9843, 2 x 10^{10}, rose-hip drink	52 21-78 (48) Rome I	4 weeks	Reduction in pain and flatulence	ND
Niv *et al.* 2005	*L. reuteri* ATCC 55730, 2 x 10^{8}(4 x 10^{8} first week), tablet	39 (46) Rome II	6 months	No effect	ND
Francavilla *et al.* 2010[1]	*L. rhamnosus* GG, 6 x 10^{9}, product not described	80 children 5-14 (FAD+IBD) Rome II	8 weeks	Reduction in frequency and severity of abdominal pain	ND
Gawronska *et al.* 2007	*L. rhamnosus* GG, 6 x 10^{9}, capsule	37 children (12) (a subset population of 104 FD/FAD/IBS subjects) Rome II	4 weeks	Moderately increase treatment success, reduced frequency of pain	ND
Baussermann *et al.* 2005	*L. rhamnosus* GG, 2 x 10^{10}, capsule	50 children 6-17 (12) Rome II	6 weeks	Reduction in perceived abdominal distension	ND
O´Sullivan *et al.* 2000	*L. rhamnosus* GG, 1 x 10^{10}, tablet	19 24-60 (39) Rome I (bloating)	8+8 weeks cross-over	A trend for a reduction in the number of unformed bowel motions for patients with IBS-D	ND
O'Mahony *et al.* 2005[1]	*L. salivarius* UCC4331 or *B. infantis* 35624, 1 x 10^{10}, malted milk drink	75 18-73 (44) Rome II	8 weeks	*B. infantis* 35624: Reduction in symptom scores; composite and individual scores for abdominal pain/discomfort, bloating/ distention, and bowel movement difficulty	ND
Choi *et al.* 2011	*Saccharomyces boulardii*, 4 x 10^{11}, capsule	67 20-65 Rome II (IBS-D+IBS-M)	4 weeks	No Effect	+

Study	Strains, daily dose (cfu), product	Number of patients, age range (mean), criteria for IBS	Duration of the treatment	Clinical outcome regarding GI symptoms and bowel habit	Effect on QOL
Studies on mixtures of probiotic strains					
Hong *et al.* 2010	*B. bifidum* BGN4, *B. lactis* AD011, *L. acidophilus* AD031, *L. casei* IBS041, 4 x 10^{10}, capsule	70 21-72 Rome III	8 weeks	Reduction in abdominal pain, defecation discomfort, and symptom scores, especially with patients with loose faeces	-
Ringel *et al.* 2011	*L. acidophilus* NCFM and *B. lactis* Bi-07, 2 x 10^{11}, capsule	33 adults Rome III (non-constipation IBS)	8 weeks	Improved symptoms of bloating	-
Williams *et al.* 2008	LAB4 [=*L. acidophilus* CUL60 (NCIMB 30157) and CUL21 (NCIMB 30156), *B. lactis* CUL34 (NCIMB 30172) and *B. bifidum* CUL20 (NCIMB 30153)], 2.5 x 10^{10}, capsule	52 39 (mean) Rome II	8 weeks	Improvement in the symptom severity score of IBS, days with pain and satisfaction with bowel habit	+
Søndergaard *et al.* 2011	*L. bulgaricus*, *Streptococcus thermophilus*, *L. paracasei* ssp. *paracasei* F19, *L. acidophilus* La5 and *B. lactis* Bb12, 7.5 x 10^{10}, acidified milk	52 29-67 Rome II	8 weeks	No effect	-
Simrén *et al.* 2009	*L. paracasei*, ssp. *paracasei* F19, *L. acidophilus* La5 *B. lactis* Bb12, 2 x 10^{10}, fermented milk	74 18-70 (42) Rome II	8 weeks	No effect	-
Saggioro *et al.* 2004	*L. plantarum* LP01 and *B. breve* BR0 or *L. plantarum* LP01 and *L. acidophilus* LA02, 5 x 10^{9}, powder	50 26-64 (40) Rome II	4 weeks	Both treated groups: The symptom severity score decreased	ND
Kajander *et al.* 2008[1]	*L. rhamnosus* GG, *L. rhamnosus* Lc705, *Propionibacterium freudenreichii* ssp. *shermanii* JS and *B. animalis* ssp. *lactis* Bb12, 1.2 x 10^{9}, milk-based drink	86 20-35 (48) Rome II	5 months	Reduction in symptom score, especially distension and abdominal pain	+

Kajander *et al.* 2005[1]	*L. rhamnosus* GG, *L. rhamnosus* Lc705, *P. freudenreichii* ssp. *shermanii* JS and *B. animalis* ssp. *lactis*, Bb99, 8-9 x 10^9, capsule	103 21-65 Rome I or II	6 months	Reduction in the total symptom score and in borborygmi	-
Choi *et al.* 2011	*S. thermophilus*, *L. acidophilus* and *B. infantis* 3.75 x 10^{12} (NamYang Dairy Products Co. Ltd) + sea tangle, radish and glasswort extracts 3.15 g, fermented milk	142 18-70 Rome III	4 weeks	Both groups: Bowel symptoms improved Fibre-containing product group: Frequency and duration of defecation and straining improved in IBS-D	ND
Guandalini *et al.* 2010	VSL#3 (=*B. longum*, *B. infantis*, *B. breve*, *L .acidophilus*, *L. casei*, *L. deldrueckii* subsp. *bulgaricus*, *L. plantarum*, *S. salivarius* subsp. *thermophilus*), 4.5 x 10^{11}, powder	59 children 5-18 (12.5) Rome II	6+6 weeks cross-over	Reduction in abdominal pain/discomfort, abdominal bloating/gassiness	+
Kim *et al.* 2005[1]	VSL#3, 4.5 x 10^{11}, powder	48 18-75 Rome II (with bloating)	8 weeks	Reduction of flatulence	ND
Kim *et al.* 2003	VSL#3, 4.5 x 10^{11}, powder	25 19-70 Rome II (IBS-D)	8 weeks	Reduction in abdominal bloating	ND

QOL, Quality of life; (+), positive effect on QOL; (-), no effect on QOL; ND, Not determined; FAD, functional abdominal pain; FD, functional dyspepsia; 1) Mechanism of action studied (in the intervention by Kajander *et al.* (2005) the study on mechanism of action was published in Kajander *et al.* (2007); 2) International Classification of Health Problems in Primary Care (ICHPPC-2) of the World Organization of National Colleges, Academies and Academic Associations of General Practitioners/ Family Physicians (WONCA).

3. AIMS OF THE STUDY

The aims of this study were to:

I Determine the bacterial phylotype content of the GI microbiota of healthy controls and IBS-D subjects applying 16S rRNA gene cloning and sequencing of %G+C fractionated faecal DNA samples pooled from several individuals (studies I and II).

II Compare the GI microbial community of IBS-D subjects with that of healthy subjects using 16S rRNA gene sequencing data (study II).

III Characterise the relationship between IBS associated GI microbiota phylotypes and self-reported IBS symptoms (study III).

IV Monitor the GI phylotypes and IBS symptoms during a multispecies probiotic intervention (study IV).

4. MATERIALS AND METHODS

4.1 Study subjects

The recruitment of healthy subjects was coordinated by the Technical Research Centre of Finland (VTT) (I and II). The IBS subjects were recruited by Valio Ltd (II, IV) and Kuopio Harjula hospital (III) (Table 5). The IBS subjects fulfilled the Rome I (III) or Rome II (II, IV) criteria. Subjects or a subset of these subjects have previously been used in the following studies: Kajander *et al.* (2005), Kajander *et al.* (2007), Kassinen *et al.* (2007), Lyra *et al.* (2009), Malinen *et al.* (2005), Maukonen *et al.* (2006b), Mättö *et al.* (2005) and Rinttilä *et al.* (2011).

The study protocols for the healthy subjects participating in studies I and II were approved by the Ethical Committee of the Technical Research Centre of Finland (VTT). The study protocols for IBS subjects in studies II and IV were approved by the Human Ethics Committee of the Joint Authority for the District Hospital of Helsinki and the Uusimaa region. The study protocol for study III was approved by the Research Ethics Committee of Kuopio University Hospital. All participants provided written informed consent and were allowed to withdraw from the study at any point.

4.2 Study methods

An overview of the methods applied in this study is given in Figure 3. The methods used are listed in Table 6. The phylogenetic analyses and library comparisons are visualised in Figure 4, while the qPCR assays are listed in Table 7.

Table 5. Subjects who participated in and completed the study.

Study	Healthy		IBS		
	No. subjects (F/M)	Age mean (range)	No. subjects (F/M)	Age mean (range)	Bowel habit[1]
I	23 (16/7)	45 (26-64)	-	-	-
II	22 a subset of study I	ND	10 (6/4), Seq[2] 12 (7/5), qPCR[2]	47 (28-62) 47 (27-62)	D: 10 D: 12
III	-	-	44 (33/11)	48 (20-72)	ND
IV	-	-	Placebo: 20 (14/6) a subset of study II	47 (24-64)	D: 8 C: 8 M: 4
			Probiotic[3]: 22 (15/7)	46 (28-63)	D: 11 C: 3 M: 8

1) Bowel habit: D, diarrhoea; C, constipation; M, mixed; ND, not determined (in study II the average age of the subjects could not be determined as the corresponding age for a missing sample could not be removed from the data). 2) Seq, subject samples used in percentage of guanine plus cytosine fractioning and sequencing; qPCR, subject samples used in quantitative real-time PCR analysis. 3) Probiotic supplementation with *Lactobacillus rhamnosus* GG (ATCC 53103), *L. rhamnosus* Lc705 (DSM 7061), *Propionibacterium freudenreichii* ssp. *shermanii* JS (DSM 7067) and *Bifidobacterium breve* Bb99 (DSM 13692).

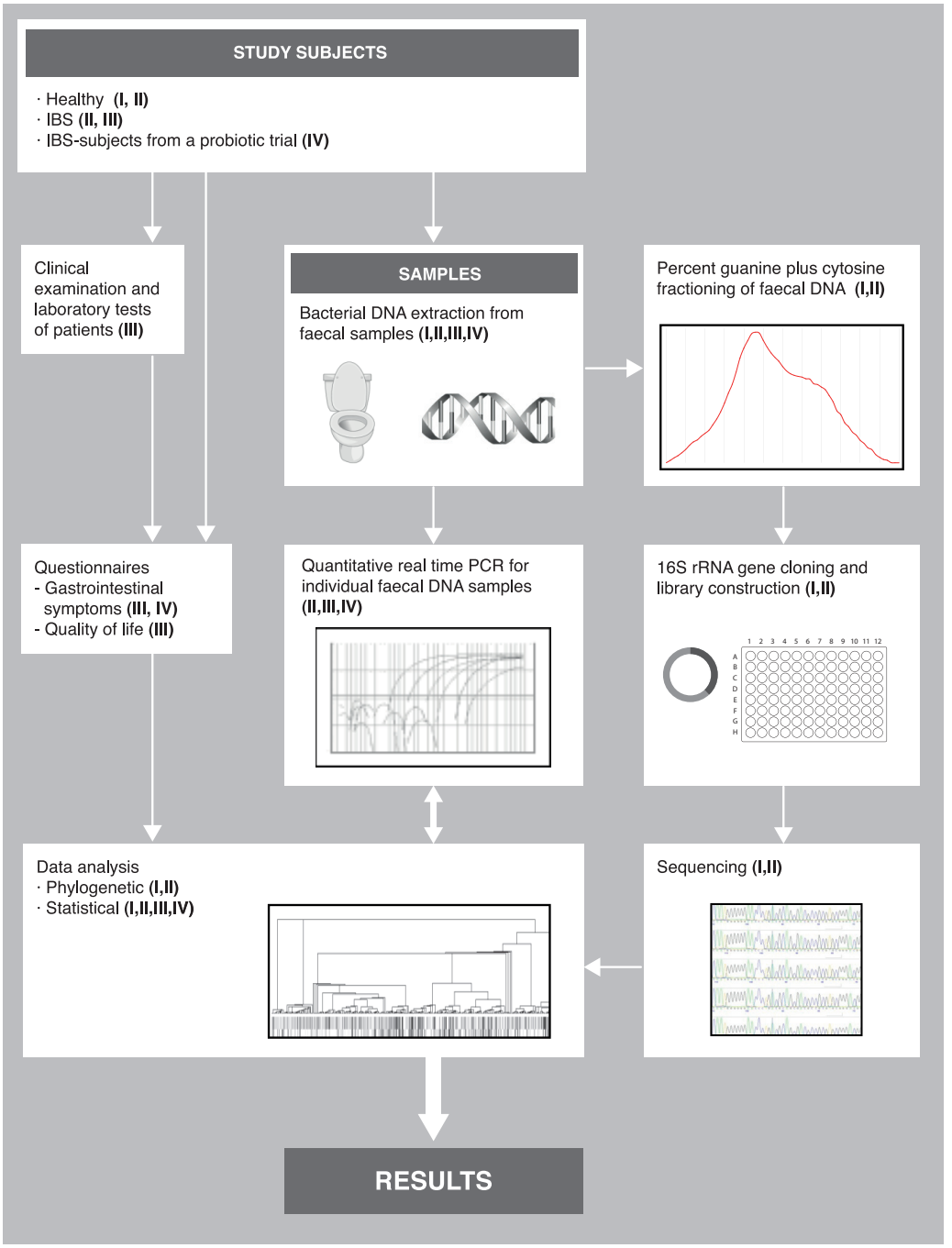


Figure 3. A flow chart of the study protocols in this thesis.

Table 6. Methods used in studies I-IV.

Laboratory methods	Study (Table)
DNA isolation from faecal samples	I, II, III, IV
DNA quantification	I, II, III, IV
%G+C profiling and fractioning in 5% intervals	I, II
Purification of %G+C fractions	I, II
16S rRNA gene clone library construction	I, II
Sequencing of clone libraries	I, II
qPCR	II, III, IV, Table 7
Other methods	**Study (Figure)**
Sequence analysis and community comparisons	I, II, Figure 4
Mann-Whitney U-test	II, III, IV
The Pearson's χ^2 test	III
PCA	III, IV
Linear models (ANOVA, F-test, t-test)	III, IV
Clinical studies and laboratory tests of patients	III
Questionnaire regarding GI symptoms	IV
Questionnaire regarding QOL and GI symptoms	III

%G+C, percentage of guanine plus cytosine; qPCR, quantitative real-time polymerase chain reaction; PCA, principal component analysis; ANOVA, analysis of variance; GI, gastrointestinal; QOL, quality of life.

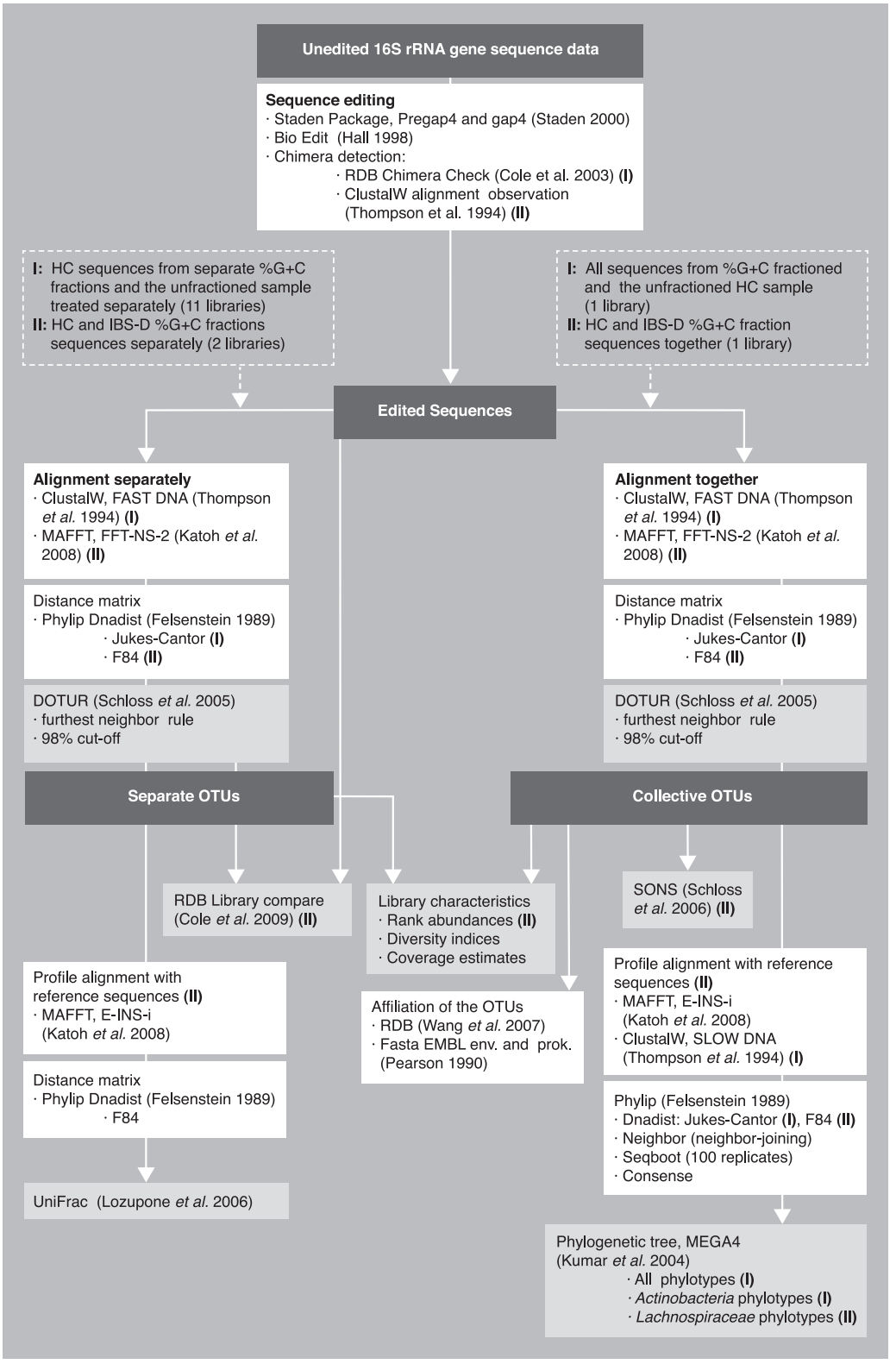

Unedited 16S rRNA gene sequence data
Sequence editing
· Staden Package, Pregap4 and gap4 (Staden 2000)
· Bio Edit (Hall 1998)
· Chimera detection:
· RDB Chimera Check (Cole et al. 2003) (I)
· ClustalW alignment observation (Thompson et al. 1994) (II)
I: HC sequences from separate %G+C fractions and the unfractioned sample treated separately (11 libraries)
II: HC and IBS-D %G+C fractions sequences separately (2 libraries)
I: All sequences from %G+C fractioned and the unfractioned HC sample (1 library)
II: HC and IBS-D %G+C fraction sequences together (1 library)
Edited Sequences
Alignment separately
· ClustalW, FAST DNA (Thompson et al. 1994) (I)
· MAFFT, FFT-NS-2 (Katoh et al. 2008) (II)
Alignment together
· ClustalW, FAST DNA (Thompson et al. 1994) (I)
· MAFFT, FFT-NS-2 (Katoh et al. 2008) (II)
Distance matrix
· Phylip Dnadist (Felsenstein 1989)
· Jukes-Cantor (I)
· F84 (II)
Distance matrix
· Phylip Dnadist (Felsenstein 1989)
· Jukes-Cantor (I)
· F84 (II)
DOTUR (Schloss et al. 2005)
· furthest neighbor rule
· 98% cut-off
DOTUR (Schloss et al. 2005)
· furthest neighbor rule
· 98% cut-off
Separate OTUs
Collective OTUs
RDB Library compare (Cole et al. 2009) (II)
Library characteristics
· Rank abundances (II)
· Diversity indices
· Coverage estimates
SONS (Schloss et al. 2006) (II)
Profile alignment with reference sequences (II)
· MAFFT, E-INS-i (Katoh et al. 2008)
Affiliation of the OTUs
· RDB (Wang et al. 2007)
· Fasta EMBL env. and prok. (Pearson 1990)
Profile alignment with reference sequences (II)
· MAFFT, E-INS-i (Katoh et al. 2008)
· ClustalW, SLOW DNA (Thompson et al. 1994) (I)
Distance matrix
· Phylip Dnadist (Felsenstein 1989)
· F84
Phylip (Felsenstein 1989)
· Dnadist: Jukes-Cantor (I), F84 (II)
· Neighbor (neighbor-joining)
· Seqboot (100 replicates)
· Consense
UniFrac (Lozupone et al. 2006)
Phylogenetic tree, MEGA4 (Kumar et al. 2004)
· All phylotypes (I)
· Actinobacteria phylotypes (I)
· Lachnospiraceae phylotypes (II)

Table 7. Group and phylotype specific real-time PCR assays applied in this study and the rationale to their use.

Assay (Phylum)[1]	Assay's reference	Association to IBS vs. HC (reference)	Study
Eggerthella lenta–like (*A*)	II	↑ IBS-D (In sequence library comparison, II)	II
Enterobacteriaceae (*P*)	II	↑ IBS-D (In sequence library comparison, II)	II
Bifidobacterium spp. (*A*)	Rinttilä *et al.* 2004	↓ IBS-D (NS, Malinen *et al.* 2005)	III
Bifidobacterium (*pseudo*)*catenulatum*-like (*A*)	Kassinen *et al.* 2007	↓ (NS, Kassinen *et al.* 2007)	III
Clostridium coccoides/ *Eubacterium rectale*-group (*F*)	Rinttilä *et al.* 2004	↓ (NS, Malinen *et al.* 2005)	III
Desulfovibrio desulfuricans-group (*P*)	Rinttilä *et al.* 2004	↓ IBS-D (NS, Malinen *et al.* 2005)	III
Eubacterial 16S (All eukaryotic phyla)	Kassinen *et al.* 2007	-	III
Lactobacillus -group (*F*)	Rinttilä *et al.* 2004	↓ IBS-D (vs. IBS-C, Malinen *et al.* 2005)	III
Streptococcus bovis -like (*F*)	Kassinen *et al.* 2007	↑ (NS, Kassinen *et al.* 2007)	III
Veillonella spp. (*F*)	Rinttilä *et al.* 2004	↑ IBS-C (Malinen *et al.* 2005)	III
Clostridium cocleatum 88% (*F*)	Kassinen *et al.* 2007	↓ (NS, Kassinen *et al.* 2007; IBS-D[1], Lyra *et al.* 2009)	III, IV
Collinsella aerofaciens-like (*A*)	Kassinen *et al.* 2007	↓ (Kassinen *et al.* 2007, IBS-D+IBS-M, Lyra *et al.* 2009)	III, IV
Coprococcus eutactus-like (*F*)	Kassinen *et al.* 2007	↓ (Kassinen *et al.* 2007)	III, IV
Ruminococcus torques 91% (*F*)	Kassinen *et al.* 2007	↑ IBS-D+IBS-M (Lyra *et al.* 2009)	III, IV
Ruminococcus torques 94% (*F*)	Kassinen *et al.* 2007	↑ (NS, Kassinen *et al.* 2007; IBS-D, Lyra *et al.* 2009)	III, IV
Bacteroides intestinalis-like (*B*)	Lyra *et al.* 2009	↓ IBS-D[2] (Lyra *et al.* 2009)	IV
Clostridium thermosuccinogenes 85% (*F*)	Lyra *et al.* 2009	↓ IBS-D[1] (Lyra *et al.* 2009)	IV
Ruminococcus torques 93% (*F*)	Lyra *et al.* 2009	↑ IBS-M (Lyra *et al.* 2009)	IV

1) The percentage indicates the 16S rRNA gene similarity to the nearest cultured bacterial species, '–like' indicates over 98% similarity. *A*, *Actinobacteria*; *B*, *Bacteroidetes; F*, *Firmicutes; P, Proteobacteria*; ↑, increase; ↓, decrease; NS, result statistically non-significant; 1, Outcome in IBS-M in addition to HC; 2, Outcome in IBS-C in addition to HC.

Figure 4. The community comparisons performed for clone library 16S rRNA gene sequences. I, Study I; II, Study II; HC, healthy controls; IBS-D, diarrhoea-predominant irritable bowel syndrome; %G+C, percent guanine plus cytosine; OTU, operative taxonomic unit.

5. RESULTS AND DISCUSSION

5.1 Community analysis of a healthy GI microbiota (I)

The microbiota of the human GI tract is characterised by an abundance of uncultured bacteria most often assigned to the phyla *Firmicutes* and *Bacteroidetes* (Figure 2). However, it has been suggested that high G+C bacteria (*Actinobacteria*) are underrepresented in many culture-independent studies (Harmsen *et al.*, 2000; Khachatryan *et al.*, 2008). To comprehensively evaluate the human intestinal microbiota across the full range of its genomic G+C content (25-75%), a %G+C profiled and fractionated faecal bacterial DNA sample pooled from 23 individuals was cloned, and the 16S rRNA genes were partially Sanger sequenced (Table 5, Figure 3, Table 6). Three fractions (%G+C 25-30, 40-45, 55-60) were initially cloned and sequenced by Kassinen *et al.* (2007), and this study completed the work on seven fractions. In the %G+C fractionation approach, the genomic DNA exposed to bisbenzimidazole is centrifuged in a caesium chloride (CsCl) gradient and thereafter fractionated. The denser DNA with a high G+C content migrates to the bottom of the CsCl gradient during high speed centrifugation, as the bisbenzimidazole preferably bind to adenine and thymine and affects the buoyant density of DNA (Holben & Harris, 1995). Partitioning of the complex microbiota into %G+C fractions with 5% increments aided in a closer observation of the genomic high %G+C bacteria. Furthermore, the method restrained the overamplification of dominant species that would have obscured the less prevalent ones. The 16S rRNA gene clones from the complete microbiota %G+C profile, were further analysed phylogenetically and statistically (Table 6, Figure 4). For comparison, an unfractionated but otherwise analogously cloned and sequenced sample was prepared from the same pool of microbiota DNA.

5.1.1 Phylum distribution

The acquired 3199 sequences from the combined fractionated sample libraries of healthy volunteers represented 455 OTUs, and the 459 sequences from the unfractionated sample represented 131 OTUs with the applied 98% sequence similarity criterion. The %G+C fractioning prior to cloning and sequencing enhanced the recovery of sequences affiliating with *Actinobacteria* (26.6%) by over seven-fold compared with the unfractionated sample (3.5%). Meanwhile, the proportion of *Firmicutes* decreased from 93.2% to 68.5% as an increase in the proportion of one phylum reduces that of the others (Table 2 in study I). The proportion of sequences affiliating with *Actinobacteria* in the unfractionated sample analysed in this study is comparable with previous estimations applying conventional 16S rRNA cloning and sequencing without %G+C fractionating, which yielded 0-6% of the detected intestinal microbiota (Figure 2). The relative proportions of less abundant *Bacteroidetes* and *Proteobacteria* in the microbiota were less affected by the %G+C fractionation of the faecal DNA sample prior to sequencing (3.1% to 2.8% and 1.3% to 0.2%, respectively) (Table 2 in study I). The phylum distribution of the combined fractions did not differ much after proportioning them according to the DNA content in each fraction (Figure 5). However, the %G+C profile fractions represent individual cloning and sequencing experiments, in which an equal number of clones

was sequenced, despite the different proportional amounts of DNA within the fractions, and quantitative conclusions should therefore be drawn with caution.

5.1.2 Observations on the %G+C fractions

The distribution of phyla between the individual clone libraries of the fractionated sample revealed that *Firmicutes* mostly settled in the lower %G+C content portion of the profile, whereas *Actinobacteria* were found in the fractions with a %G+C content ranging from 50% to 70% (Figure 5). Sequences in these fractions also had the highest %G+C contents in their 16S rRNA gene sequences, ranging from 58% to 62% (Table 1 in study I). In fact, %G+C fractions 30-50 yielded a similar phylotype distribution to the unfractionated library, were dominated by the *Firmicutes* (*Clostridium* clusters XIV and IV), and accounted for 54% of the profiled DNA. However, %G+C fractions 50-70 were dominated by *Actinobacteria*, comprising 41% of the total DNA in the original fractionated sample (Figure 5). %G+C fractions 50-60 had a comparatively lower diversity and a higher abundance with bifidobacteria than %G+C fraction

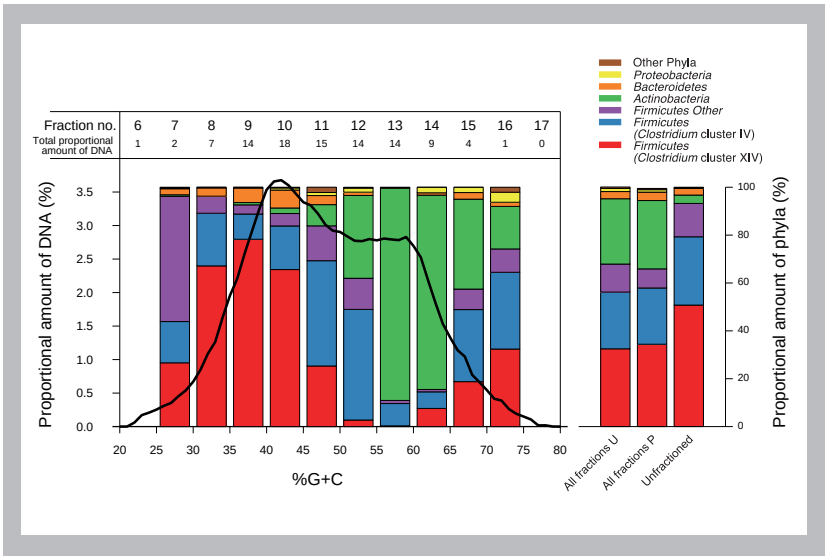


Figure 5. The %G+C profile of pooled faecal microbial DNA from healthy subjects and the distribution of bacterial phyla in separate %G+C fractions and in the unfractionated DNA sample pool. The phyla were determined according to the the Ribosomal Database Project Classifier (Wang *et al.*, 2007) with a bootstrap confidence level of 80%. The *Clostridium* clusters were determined by a combined Clustal W alignment (Thompson *et al.*, 1994) with reference sequences classified into 16S rRNA gene clusters according to Collins *et al.* (1994). The distribution of the *Clostridium* clusters IV and XIV within the *Firmicutes* was represented by 23.5% and 33.0%, respectively, of the total sequences in the unproportioned count. Whereas, the actinobacterial phylotypes consisted of the orders *Bifidobacteriales*, *Coriobacteriales* and *Actinomycetales*, accounting for 12.4%, 13.4% and 0.8% of the sequences, respectively. U, Phyla unpropotioned with DNA amount in %G+C fractions; P, Phyla proportioned with DNA amount in %G+C fractions.

45-50, with sequences affiliating mainly with *Clostridium* clusters IV and XIV and having a higher diversity according to Shannon entropies (Additional file 2 in study I). This could be due to the higher heterogenity among clostridia and more uniform bifidobacterial 16S rRNA gene sequences compared to clostridia. According to SONS analysis (Schloss & Handelsman, 2005), at least 80% of sequences from low %G+C fractions (30-40%) were shared with the unfractionated sample. However, in the high %G+C content fractions (55-65%), the proportion of shared sequences was only 33%. This further supports the contention that the sequences from unfractionated community samples mostly represent phylotypes from low G+C bacteria.

5.1.3 Methodological findings

In this study, the *Actinobacteria* outnumbered *Bacteroidetes* as in the pyrosequencing study by Andersson *et al.* (2008) and the metagenomic study by Gill *et al.* (2006), in which no *Bacteroidetes* at all were found. A technical point conserning the outcome is that the rigorous DNA extraction method used in this study may have raised the DNA yield from robust Gram-positive bacteria and thus lowered the proportion of DNA from more easily lysed Gram-negative bacteria (*Bacteroides*) in both fractionated and unfractionated samples. However, also a delay or inadequate temperature in freezing may lower the proportion of *Bacteroidetes* detected, as discussed in Salonen *et al.* (2010).

The unfavourable PCR reaction conditions involved in the amplification of a highly diverse community sample like the GI microbiota is one cause for the discrimination of *Actionobacteria* in many studies as discussed in section 2.2. As an example, a comparative study of a metagenomic library and a library constructed from 16S rRNA gene clones showed that *Actinobacteria* were more abundant in the former (16% vs. 6%), in which PCR bias did not affect the outcome (Manichanh *et al.*, 2008). Moreover, an unexpectedly low frequency and abundance of bifidobacteria in infant faecal microbiotas possibly due to methodological limitations was observed in a study by Palmer *et al.* (2007). It is generally known that DNA with a high G+C content is amplified less efficiently compared to the DNA with a lower G+C content. Primers and other factors in a PCR reaction may also cause a bias as, for example, 'universal' primers may discriminate *Actinobacteria* (Farris & Olson, 2007). This was overcome in Hill *et al.* (2010) using the *cpn*60 gene as a phylogenetic marker. It is thus good to question the predominant use of the 16S rRNA gene in the phylogenetics of microbial communities.

The sequencing step in the community analysis may also introduce errors in the community structure. In this study, the sequencing conditions had to be modified for several clones in the high %G+C fractions (60-70% G+C content). These clones were members of the order *Coriobacteriales* and had an extremely G+C-rich region in which the sequencing reaction had stopped. In a high throughput community analysis, these kind of clones can easily be hidden, as the 'optimal' conditions are less optimal for the high G+C bacteria, which have thus been rare or absent in earlier 16S rRNA gene-based clone libraries of the intestinal microbiota. Indeed, over half of the actinobacterial OTUs in our study belonged to the order *Coriobacteriales* after dimethyl sulphoxide treatment of the sequencing reaction mixtures, which was used to prevent self-complementarity and loop formation in G+C-rich DNA regions.

5.1.4 Abundance of *Coriobacteriales*

Bifidobacteria (order *Bifidobacteriale*s) have received considerable attention in research on the GI microbiota, due to their proven beneficial effects in hosts. Therefore, it is somewhat surprising that a large proportion of GI community analysis reports have used methods that overlook them (Figure 2). Propionibacteria (order *Actinomycetales*) also display probiotic properties (Cousin *et al.*, 2010). In this respect, the probiotic potential of the third actinobacterial order within the GI tract (*Coriobacteriales*), which is phylogenetically close to the previous ones and highly abundant according to this study (Figures 2 and 3 in study I), warrants attention.

Previous studies have demonstrated that low abundances of members of the family *Coriobacteriaceae* within the *Actinobacteria* have been associated with a high risk of colon cancer (*Collinsella aerofaciens*) (Moore & Moore, 1995), CD (*Atopobium* group) (Manichanh *et al.*, 2006), IBS (*C. aerofaciens*–like phylotype) (Kassinen *et al.*, 2007) and a familial disease causing Mediterranean fever (*Atopobium* group) (Khachatryan *et al.*, 2008). Therefore, in addition to the more intensively studied bifidobacteria and propionibacteria, other actinobacterial genera such as *Coriobacteriaceae* may also be associated with the health status. However, more detailed data are required to assess their role in health and disease. Moreover, Jakobsson *et al.* (2010) recorded a dramatic decline in *Actinobacteria* after clarithromycin and metronidazole treatment while Mäkivuokko *et al.* (2009) reported reduced quantities of *C. aerofaciens* in elderly subjects using non-steroidal anti inflammatory drugs (NSAIDs). In addition, newborns from mothers treated with antibiotics during pregnancy or born by Caesarean section had lower proportions of members of *Atopobium* cluster in their gut microbiota (Fallani *et al.*, 2010).

Finns and Swedes both have high levels of *Actinobacteria* (Mueller *et al.*, 2006; Andersson *et al.*, 2008; Jakobsson *et al.*, 2010) (study I). Similarities in dietary habits, could explain such trends. *C. aerofaciens* has been shown to decrease in subjects on a reduced carbohydrate weight loss diet (Walker *et al.*, 2011b), while the consumption of galacto-oligosaccharides (GOS) or fructo-oligosaccharides (FOS) has been reported to increase *Bifidobacterium adolescentis* and *C. aerofaciens* activity in the gut (Tannock *et al.*, 2004). Moreover, children in a rural African village with a high fibre diet have been shown to have increased overall levels of *Actinobacteria* compared to Europeans (De Filippo *et al.*, 2010). In addition to increased activity, a diet rich in GOS and FOS could in the long term increase the counts of *Actinobacteria*. A study on European infants has shown that a north-south gradient exist with a higher proportions of *Bifidobacteria* spp. and *Atopobium* cluster in the north (the most northern and southern study populations were Swedes and Spanish, respectively) (Fallani *et al.*, 2010). It is noteworthy that in Scandinavian countries breast-feeding rates are high and infants are weaned late (Yngve & Sjöström, 2001), which elevates the count of *Bifidobacteria* spp. and *Atopobium* cluster bacteria (Fallani *et al.*, 2010). Notably, two studies on Swedish subjects were conducted by the same research group and the microbial DNA isolation method described was the same (Andersson *et al.*, 2008; Jakobsson *et al.*, 2010), thus strongly influencing the phylum-level outcome of the studies (Salonen *et al.*, 2010b). The used methodology may also be a significant issue in the other large-scale sequencing studies on microbiota

performed in same laboratories and reporting low amounts of *Actinobacteria* in the GI microbiota of healthy individuals (Figure 2).

5.2 Comparison of the GI microbiota of IBS-D and healthy individuals (II)

The GI microbiota in IBS-D differs from other IBS symptom subtypes and has many special features, as reviewed in section 2.3. In this study, the GI microbiota of IBS-D subjects was analysed and the microbial community was compared with that of healthy controls (HC). The libraries were constructed analogously by using %G+C profiling and fractionation combined with 16S rRNA gene clone library sequencing, as described in study I (Table 5, Figure 3, Table 6, Figure 4). As in study I, three fractions (%G+C 25-30, 40-45, 55-60) were initially cloned and sequenced by Kassinen *et al.* (2007), and this study completed the sequencing work. Furthermore, a closer quantification of an interesting phylotype (*Eggerthella lenta*–like) and group (*Enterobacteriaceae*) was performed with qPCR on the individual samples (Table 7).

5.2.1 Characteristics of the libraries

The pooled sample from IBS-D patients (n = 10) resulted in a total of 3267 sequences and 302 OTUs with the applied 98% cut-off level for OTUs. The set was compared to the pooled sample of healthy controls (n = 23) that comprised 3199 sequences and substantially more OTUs, in total 428 (Table 1 in study II). The sequence pool from both community samples altogether made up 578 OTUs, of which 30.4% were shared. The portion of sequences shared between the two samples was 81.0% (study II, Figure 4). According to Good's formula (Good, 1953), the coverage of clone libraries was above 95%, which is much lower than in pyrosequencing studies (Andersson *et al.*, 2008), but the same magnitude was observed in both groups, which is important in a comparative study (Table 1 in study II). The IBS-D library contained fewer unique sequences and OTUs than the HC library (Figure 4 in study II). In addition to a lower quantity of OTUs, lower Shannon and Simpson indices for diversity as well as Chao and ACE richness estimates were observed in the IBS-D library compared to the HC library (Table 1 in study II). The lower number of individuals in the IBS-D sample most likely caused at least part of the lower diversity and uniqueness in IBS-D sequence library as well as bowel habit in IBS-D, as diarrhoea itself has been shown to disrupt the microbial ecology and reduce the overall microbial count in the bowel (Mai *et al.*, 2006). Diversity in any ecosystem is generally thought to be important, as it creates stability and prevents dominance by a single species. It also helps in coping during stressful events in the ecosystem, as several species can handle same tasks. A reduced bacterial diversity has very recently been detected also in another IBS-D cohort (Carroll *et al.*, 2011) as well as in a stydy with unsubtyped IBS patients (Noor *et al.*, 2010), in inflammatory intestinal diseases like such as CD (Seksik *et al.*, 2003; Dicksved *et al.*, 2008), and in infants with NEC (Wang *et al.*, 2009). The rank abundance curves of the libraries showed highly similar OTU evenness and therefore a similar community structure in terms of the number of OTUs and the sequence count within OTUs, but did not indicate the type of species they represented (Figure 2 in study II).

5.2.2 Phylum level differences

The phylum-level microbial community comparison with the Ribosomal Database Project (RDP) classifier (Cole *et al.*, 2009) revealed

that the IBS-D library had significantly more sequence representatives of *Proteobacteria* and *Firmicutes* than the HC library, and fewer representatives of *Actinobacteria* and *Bacteroidetes* (Figure 3 in study II). The finding concerning the three latter phyla is in accordance with a cohort of all IBS symptom subtypes by Rajilić-Stojanović *et al.* (2011). Furthermore, the comparison made with OTUs revealed that the IBS-D library was significantly richer in *Firmicutes* than the HC library. Differences at such a high taxonomic level are less prone to be biased by differences in the subject number. The number of sequences of the phylum *Bacteroidetes* was lower in the IBS-D than the HC library, but low overall in both libraries. This was possibly due to technical issues, as discussed in study I.

5.2.3 Core phylotypes

Tap *et al.* (2010) determined the core OTUs among 17 subjects (vegetarians and omnivores from the Netherlands and France) with a criterion of 50% of the subjects harbouring the OTU. The 578 common OTUs for IBS-D and HC in this study were compared with full-length public database representatives of the 66 core OTUs (*Firmicutes* 57, *Bacteroidetes* 7, *Actinobacteria* 2) determined by Tap *et al.* (2010). As a result, 57 of the 66 core OTUs were detected among the common OTUs in this study. Of the core OTUs detected in this study, one and ten OTUs had sequence representatives in only the IBS-D or HC library, respectively, suggesting that the IBS-D library is less diverse and resilient to core species.

5.2.4 Lachnospiraceae

A marked proportion of the sequences and OTUs in IBS-D (45% and 41%) and HC (33% and 30%) libraries affiliated with the family *Lachnospiraceae*, which diverged significantly between the community samples in the RDP Library Compare analysis (Additional file 3 and 4 in study III). Related to this finding, serum antibodies to certain flagellins that are expressed by *Clostridium* XIV bacteria are elevated in CD (Lodes *et al.*, 2004). One of these antibodies had a high amino acid similarity with a flagellin gene belonging to *Clostridium* cluster XIV bacteria (Duck *et al.*, 2007). Antibodies against these bacterial flagellins were found more frequently in patients with IBS, especially PI-IBS patients, making an interesting link between IBS and CD (Schoepfer *et al.*, 2008). The abundance of *Lachnospiraceae* in IBS-D in this study supports this finding, although the result does not provide any more specific qualitative information on the appearance of this group within the IBS-D and HC GI communities. As pointed out by Salonen *et al.* (2010), *Lachnospiraceae* was increased in patients with *Clostridium difficile* associated diarrhoea (CDAD), but not in IBD although these three diarrhea-causing GI conditions shared many similarities in the GI microbiota (Frank *et al.*, 2007; Khoruts *et al.*, 2010). Previously, the *Clostridium coccoides - Eubacterium rectale* group (*Clostridium* cluster XIV) has been detected in IBS patients with contradictory results (Malinen *et al.*, 2005; Mättö *et al.*, 2005) (Table 3). The reason for this discrepancy is most probably that the group is too broad to be analysed with one assay.

5.2.5 Quantitative real-time PCR

Altogether 130 and 8 *Gammaproteobacteria* sequences were seen in IBS-D and HC clone libraries, respectively (Additional file 4 in study II). Moreover, a single phylotype seemed to dominate in IBS-D sample (Figure 1 in study II). Therefore, a qPCR assay was developed and used to identify this *Enterobacteriaceae* –like phylotype (Table 7). An

increase was seen in the *Enterobacteriaceae* phylotype in IBS-D, although it was nonsignificant (p = 0.28) (Figure 6A in study II). The %G+C fractionation enabled the observation of differences in *Gammaproteobacteria* between HC and IBS-D. Only one sequence of this class was obtained in the unfractionated library of HC in study I, whereas the fractionated sample also contained sequences of other classes of *Proteobacteria* (Table 2 in study I). *Proteobacteria* are clinically relevant bacteria, since they include many pathogens or opportunistic pathogens. However, the pathogenicity of gammaproteobacterial strains cannot be determined from the 16S rRNA gene. Kerckhoffs *et al.* (2010) suggested *Pseudomonas aeruginosa* to have a role in the pathophysiology of IBS, as this *Gammaproteobacteria* was found more frequently and at higher abundances in small intestinal brush samples and faeces of all IBS patient groups than in healthy subjects. Interestingly, increase of *Gammaproteobacteria* was recently also detected in children with IBS (Saulnier *et al.*, 2011) and the abundance was found to be associated with IBS-symptoms in adults (Rajilić-Stojanović *et al.*, 2011).

In accordance with this study, also CD is associated with the abundance of enterobacteria and gammaproteobacteria (Seksik *et al.*, 2003; Walker *et al.*, 2011a), including opportunistic pathogens (Martinez-Medina *et al.*, 2006), such as *Pseudomonas areuginosa* in IBS (Kerckhoffs *et al.*, 2010). Interestingly, increased expression levels of TLR-4 that recognise lipopolysaccharides of Gram-negative bacteria were observed in IBS patients' biopsy samples, although in lower amounts than in those of IBD patients' (Brint *et al.*, 2011). Furthermore, *Gammaproteobacteria* are abundant in post-gastric bypass individuals (Zhang *et al.*, 2009) and in preterm infants with NEC (Wang *et al.*, 2009). In addition, a lower abundance of *Enterobacteriaceae* within the *Gammaproteobacteria* and lower levels of SCFAs have been observed in EU children compared to children in a rural African village on a fibre-rich diet (De Filippo *et al.*, 2010). Thus, the abundance of *Gammaproteobacteria* can be an indicator of an unfavourable or distorted microbiota in many conditions. Lupp *et al.* (2007) demonstrated that inflammation supported the growth of aerobic bacteria in mice, especially *Enterobacteriaceae*, and that the simultaneous precence of an enteropathogen and inflammation reduced the number of colonic bacteria. The low diversity of the whole faecal microbiota and high abundance of enterobacteria in IBS-D in this study may thus reflect inflammation in IBS.

Furthermore, an *Eggerthella lenta*–like phylotype was quantified with qPCR (Table 7), since the genus seemed to be significantly elevated in IBS-D samples (Additional file 4 in study II) according to the RDP Library Compare analysis (Cole *et al.*, 2009). The result revealed an opposite though nonsignificant (P = 0.42) trend (Figure 6B in study II), and was thus in accordance with the association of the healthy microbiota with *Coriobacteriaceae* (see study I). This observation demonstrated the importance of analysing individual samples to obtain quantitative data.

5.3 GI symptom and microbiota in IBS (III)

Abdominal pain or discomfort is the hallmark symptoms of IBS. The symptoms may improve with defecation or their onset can be associated with alterations in stool form and frequency (Longstreth *et al.*, 2006). Other commonly reported symptoms include urgency and straining during defecation, bloating, mucus in the stool and the feeling of incom-

plete evacuation (Khan & Chang, 2010). As the GI microbiota has been shown to be altered in IBS (see section 2.3.2.4), it is reasonable to hypothesise that there is a link between the GI microbiota and GI symptoms that lead to impaired QOL (Khan & Chang, 2010). Therefore, the association of certain bacterial groups or phylotypes previously linked to either the microbiota of IBS subjects or healthy controls and self-reported symptoms experienced by IBS subjects were examined in study III (Table 5, Table 7). The composition of the GI microbiota was investigated with thirteen qPCR assays, after which associations between quantities or prevalences of the selected bacterial groups or phylotypes and various IBS-related symptoms experienced by the patients were investigated.

5.3.1 Microbiota and GI symptoms

Visualisation of the principal component analysis (PCA) results revealed a positive association between the *Ruminococcus torques* 94% phylotype (94% 16S rRNA gene sequence similarity to *R. torques*) and the bowel symptoms, both separately and jointly (Figure 1A and B in study III). Abdominal pain, which is the keystone symptom in IBS, associated most strongly with *R. torques* 94% among the individual bowel symptoms in the PCA (study III, Figure 1b). Furthermore, the presence and abundance of *R. torques* 94% was linked to the severity of bowel symptoms (study III, Table 7). Previously, *R. torques* 94% has been associated with IBS-D (Lyra *et al.*, 2009). *R. torques* belongs to the *Clostridium coccoides* group (XIV), which is generally thought to be the most prevalent group in the gut. Unlike many other members of *C. coccoides* group XIV, *R. torques* is not a beneficial butyrate producer (Dethlefsen *et al.*, 2006). Instead, *R. torques* efficiently degrades mucin and has been associated with CD (Martinez-Medina *et al.*, 2006; Png *et al.*, 2010; Joossens *et al.*, 2011), as has the phylotype *R. torques* 94% (Frank *et al.*, 2007). The passage of mucus is a reported symptom in IBS that could be linked to the abundance of *R. torques* 94%, but was not inquired from the participants in this study. Bacterial flagellins possibly play a role in IBS aetiology as they provoke antibody (Schoepfer *et al.*, 2008) and TLR-5 (flagellin recognition) expression in IBS patients (Brint *et al.*, 2011). Moreover, the presence of *R. torques* 94% had a negative effect on the abundance of *Coprococcus eutactus* 97%, *Collinsella aerofaciens*–like, and *Clostridium cocleatum* 88% phylotypes according to linear models (study III, Table 7) with all three latter phylotypes being associated with healthy microbiota (Kassinen *et al.*, 2007; Lyra *et al.*, 2009) (Table 7). Thus, the proportions of intestinal bacteria may reflect the nature of the microbial interactions in the gut and could thus be even more significant in IBS than the absolute number of certain phylotypes.

A weaker negative association was observed for *Bifidobacterium* spp., *C. aerofaciens*–like, *C. eutactus* 97%, *D. desulfuricans* –group, *Lactobacillus* spp., *Veillonella* spp. and the bowel symptoms or symptom group scores in PCA visualisation (Figure 1A and B in study III). All these except for *Veillonella* spp., which have been detected in highest quantities among IBS-C patients, have previously been decreased in IBS-D patients in comparison to other IBS symptom subtypes and healthy control subjects (Kassinen *et al.*, 2007; Lyra *et al.*, 2009) (Table 7). The patients were not grouped according to the main bowel habit in study III. However, they were mostly suffering from IBS-D and IBS-M (Atte von Wright, personal communication). Very recently, the abundance of *Bifidobacteria* has been shown

to be negatively associated with pain in healthy individuals (Jalanka-Tuovinen *et al.*, 2011) as well as with intestinal symptoms in IBS patiens (Rajilić-Stojanović *et al.*, 2011).

5.3.2 Microbiota and body mass index (BMI)

The average BMI (26.3) of the participants indicated mild overweight and thus the participants were divided to two groups, under and above a BMI of 25, for further analysis. The group with a BMI over 25 had more *Bifidobacterium* spp. ($P = 0.009$) and nearly statistically significantly less *Lactobacillus* spp. ($P = 0.060$), and also reported more systemic symptoms than the normal weight subjects (Table 5 in study III). Higher counts of *Bifidobacteria* spp. have also been observed in another study with overweight subjects (Turnbaugh *et al.*, 2009). Furthermore, an energy restricted diet was found to reduce the number of *Bifidobacterium longum* and *Bifidobacterium adolescentis* and increase that of *Lactobacillus* group (Santacruz *et al.*, 2009). Species-specific assays could thus have revealed more specific alterations in the context of overweight and bifidobacteria, although this was not originally an aim of the study.

Earlier, a reduced prevalence and abundance of the *Collinsella aerofaciens* phylotype was shown in IBS patients compared to healthy subjects, as well as an increase in the prevalence and count of *R. torques* 94% (Kassinen *et al.*, 2007; Lyra *et al.*, 2009) (Table 7). An interesting finding for *C. aerofaciens*, in addition to the ones discussed in the context of a healthy microbiota in study I, was that the phylotype was negatively associated with *R. torques* 94% (Figure 1 in study III). Also, in a recent study, patients with CD and their relatives had more *R. torques* and less *C. aerofaciens* in their faeces than healthy controls (Joossens *et al.*, 2011). Furthermore, in this study, *C. aerofaciens* was associated with a low blood pressure, low sugar levels, low BMI and high numberts of *Coprococcus eutactus* 97%. In fact, *C. aerofaciens* was absent in subjects with a BMI over 30. Increased systemic symptoms experienced by overweight subjects (BMI > 25) make it difficult to define the role of *C. aerofaciens* in IBS. Recently, a beneficial synbiontic effect of prebiotics and probiotics on intestinal microbiota and GI symptoms in IBS was demonstrated, as the consumption of certain oligosaccharides alone or in combination with *Bifidobacterium animalis* DN-173010 increased *C. aerofaciens* in the gut and thereby reduced bloating in patients with IBS-C (Veiga *et al.*, 2010). This independent observation strengthens the putative beneficial role of the *C. aerofaciens* phylotype in IBS symptoms in this study.

5.4 Effect of a multispecies probiotic intervention on selected GI phylotypes (IV)

The consumption of certain probiotics has been shown to alleviate the symptoms of IBS in previous studies, as reviewed in section 2.4.3. The effect of a multispecies probiotic supplement on the GI microbiota of IBS patients was assessed in a placebo-controlled six-month double-blind study. The supplement, which consisted of *Lactobacillus rhamnosus* GG, *L. rhamnosus* Lc705, *Propionibacterium freudenreichii* ssp. *shermanii* JS and *Bifidobacterium breve* Bb99, has previously been shown to alleviate the GI symptoms in IBS, and the cohort has been monitored with qPCR, but the targeted microbial groups and species were not associated with the ameliorating effect (Kajander *et al.*, 2005; Kajander *et al.*, 2007). The eight targeted phylotypes measured in this study have according to pre-

vious studies diverged between IBS symptom subtypes and healthy individuals (Kassinen *et al.*, 2007; Lyra *et al.*, 2009) (Table 7), and were thus selected for further monitoring in the probiotic trial in this study on 42 participants (Table 5).

5.4.1 GI Symptoms

At the beginning of the study, when no supplements were taken, the placebo and probiotic groups overlapped when visualised with PCA, and no clear associations were seen between the measured phylotypes and the GI symptoms (abdominal pain, borborygmi, distension, flatulence) (Figure 1A in study IV). The analogous GI symptoms in study III with those recorded in study IV were pain, bloating and gas. However, no significant positive and negative association between GI symptoms and *R.torques* 94% and the *C. aerofaciens* phylotype, respectively, was seen as in study IV. However, during the supplementation (combined time points at 3 and 6 months), the placebo group shifted in the direction of GI symptoms and the *R. torques* 94% phylotype. Furthermore, the probiotic group and the *C. thermosuccinogenes* 85% and *R. torques* 93% phylotypes shifted in the opposite direction (Figure 1B in study IV). *R. torques* 94% and *C. aerofaciens* were oriented in opposite directions at both observation points, which is in accordance with study III.

5.4.2 Phylotype alterations

Linear model comparisons showed a decrease in the quantity of *R. torques* 94% in the probiotic group ($P = 0.02$ at 6 months), whereas *C. thermosuccinogenes* 85% was elevated in the probiotic group ($P = 0.00$ and $P = 0.02$ at 3 months and 6 months, respectively). The abundance of *R. torques* 93% was higher in the probiotic group during consumption of the probiotic ($P = 0.00$ and $P = 0.00$ at 3 months and 6 months, respectively), but the detected alterations were due to a decrease in the placebo group (Table 3 and Figure 2 in study IV). The analyses for probiotic and placebo effects were also performed separately for the IBS-D symptom subtype. The IBS-C and IBS-M consisted of unbalanced and small numbers of subjects (11 and 12 respectively), and were thus inappropriate for statistical analysis as separate groups. Before the consumption of the probiotic supplement, the *R. torques* 94% phylotype was more abundant in the placebo group than in the probiotic group among IBS-D patients ($P = 0.04$ at 0 months). The level decreased among the probiotic-consuming IBS-D subjects in 6 months ($P = 0.01$ at 6 months). The levels of *C. thermosuccinogenes* 85% phylotype among IBS-D subjects increased throughout the intervention in the probiotic-receiving group ($P = 0.01$ at 3 months and $P = 0.05$ at 6 months). *R. torques* 93% showed higher levels in the probiotic group at the 3-month time point ($P = 0.00$) among IBS-D patients.

The fluctuation in the counts of the phylotypes between time points could be within the normal temporal variation of the intestinal microbiota, which is especially unstable in IBS patients (Maukonen *et al.*, 2006a). Although the subjects were also told not to use other probiotics during the study, the previous and in some cases long-term consumption of probiotics could still have affected the microbiota of the participants, at least at the beginning of the intervention. Participating in a nutrition trial could also affect the eating habits and thereby the diet of some individuals, which would influence the microbiota (Louis *et al.*, 2007). Furthermore, stress, which is common in IBS, may alter the microbiota (O'Mahony *et al.*, 2009).

The *C. thermosuccinogenes* 85% phylotype, which increased with multispecies probiotic supplementation in this study, was previously more strongly associated with IBS-M subjects and healthy controls than with patients suffering from IBS-D (Lyra *et al.*, 2009) (Table 7). The phylotype has also occurred in other GI microbiota cohorts of healthy individuals (Eckburg *et al.*, 2005; Gill *et al.*, 2006). The *R. torques* 94% phylotype, which decreased following probiotic supplementation, has been detected at higher levels in IBS-D subjects than controls (Lyra *et al.*, 2009) (Table 7), as well as being associated with the severity of GI symptoms in study III. Thus, these alterations detected during the intervention showed a trend towards the pattern previously detected in non-IBS controls free of GI symptoms. The *R. torques* 91% phylotype has been associated with IBS-D and IBS-M and the *R. torques* 93% phylotype has been associated more strongly with healthy control subjects than with IBS-M sufferers (Lyra *et al.*, 2009) (Table 7). The naming of the phylotypes *R. torques* 91%, 93% and 94% is somewhat misleading, as they share less than genus-level similarities among each other according to sequence comparison, and the percentage refers to the similarity to the target sequence of the species *R. torques*. Thus, the bacterial species detected by the assays may have different metabolic and functional roles in the gut, as their abundance responded differently during the intervention.

5.4.3 Mucus

Some microbial alterations related to IBS may be associated with excess mucus commonly present in IBS (Longstreth *et al.*, 2006). The putatively mucin-degrading *R. torques* 94%, abundant in IBS, could serve as marker of the IBS status in the gut. Similar marker-phylotypes could be found among *Enterobacteria*. For instance, *Enterobacteria* include opportunistic pathogens, such as *Pseudomonas aeruginosa* which has proteolytic activity and is capable of degrading mucin (Aristoteli & Willcox, 2003), and has been detected at a higher prevalence and abundance in IBS (Kerckhoffs *et al.*, 2010). An excess of degraded mucin could result in a vicious circle by making the gut an even more favourable environment for the above-mentioned bacteria to grow in, and further imbalancing the microbiota and accelerating the degradation of mucin layers with a synergistic effect. This is an interesting issue to speculate as activation of mucin-producing genes in the gut has been observed in IBS (Aerssens *et al.*, 2008). Many individual studies with different cohorts and methods have reported *Gammaproteobacteria* (*Enterobacteria*) and *Clostridium* cluster XIV (*Ruminococcus torques* affiliation) to be elevated in IBS and IBD (see sections 2.3.3 and 5.2). There might be an association between the impaired GI health and the two groups of bacteria.

On the other hand, as discussed in study IV, one mechanism of action of the probiotics in this study could be speculated to lead to mucus production, as certain probiotics promote mucin secretion *in vivo* (Ohland & Macnaughton, 2010). The expression of mucus could thus maintain the epithelial barrier by preventing the mucolytic bacteria from disrupting the mucus layer and further making the gut susceptible to other unfavourable bacteria, promoting overall dysbiosis and even low grade inflammation. Another potential mechanism of action of the probiotic mixture could be associated with the production of lactic acid, which can disrupt the outer membranes of Gram-negative pathogens such as *P.*

aeruginosa and thus inhibit their growth (Alakomi *et al.*, 2000).

The findings of this study support the contention that a multispecies probiotic supplementation has a favourable effect on the overall GI microbiota of IBS patients, as in a study by Kajander *et al.* (2008). In the studies of this thesis the same probiotic strains were used, with the exception that *Bifidobacterium animalis* ssp. Bb12 was used instead of *Bifidobacterium breve* Bb99 in study IV (original intervention published by Kajander *et al.* 2005). The effects of probiotic strains or their combinations are unique, and thus a change in even one strain of a mixture may alter the outcome in the alleviation of the symptoms and modulation of the microbiota (Spiller, 2008).

6. CONCLUSIONS

I %G+C profiling and fractionation prior to cloning and sequencing can reveal a significantly larger proportion of bacteria with a high G+C content among the clones recovered compared with an unfractionated sample from the human GI tract. The order *Coriobacteriales* within the phylum *Actinobacteria*, in particular, was found to be more abundant than previously estimated in conventional sequencing studies. Constant underestimation of the high G+C Gram-positive bacteria might lead to misunderstanding of their role in the healthy and diseased gut. Other members within *Actinobacteria* than bifidobacteria could be emphasised in GI microbiota studies.

II Significant differences were found between 16S rRNA gene clone libraries of IBS-D patients and controls constructed from pooled and %G+C-fractionated faecal DNA. The overall structure of the microbial community of the libraries was similar. However, the microbial communities of IBS-D patients were enriched in *Firmicutes* and *Proteobacteria*, but reduced in the number of *Actinobacteria* and *Bacteroidetes* compared to healthy individuals. Sequences and OTUs affiliating with the family *Lachnospiraceae* within the phylum *Firmicutes* were abundant in IBS-D. Individual samples showed a trend of elevated levels of the *Gammaproteobacteria* phylotype in IBS-D samples.

III An association was detected between the presence and abundance of *Ruminococcus torques* 94% and bowel symptoms in IBS subjects. Furthermore, the presence of *R. torques* 94% negatively affected the abundance of *Coprococcus eutactus* 97%, *Collinsella aerofaciens*-like and *Clostridium cocleatum* 88% phylotypes. Elevated BMI was associated with low levels of the *Collinsella aerofaciens*-like phylotype and *Lactobacillus* spp. and with high levels of *Bifidobacterium* spp.

IV The multispecies probiotic supplement with *Lactobacillus rhamnosus* GG, *L. rhamnosus* Lc705, *Propionibacterium freudenreichii* ssp. *shermanii* JS and *Bifidobacterium breve* Bb99, which is capable of alleviating IBS symptoms, shifted the intestinal microbiota of IBS subjects towards that associated with healthy controls. The abundance of the *R. torques* 94% phylotype was reduced and that of the *Clostridium thermosuccinogenes* 85%-like phylotype was increased in the probiotic-receiving group.

7. FUTURE PROSPECTS

In community analysis of the GI microbiota, methods that do not discriminate high G+C bacteria should be used. Such methods, applying for instance microarray and pyrosequencing, could be further developed. This is especially the case if IBS is most likely associated with dysbiosis of the microbiota rather than the effect of a single bacteria species. Such approaches would also aid in studying the relative proportions of different bacterial groups and phylotypes in more detail.

The symptoms have so far been the only criteria for IBS diagnosis. The *Lachnospiraceae* family within *Firmicutes* and phylotypes phylogenetically close to *Ruminococcus torques* warrant further studies as putative markers and causative agents. Attempts to cultivate *R. torques* 94% are necessary, e.g. for further *in vitro* studies on mucosal degradation, adhesion, metabolic activity, and properties such as existence of flagella in these bacteria. The cultivation and study of the role of the *Clostridium thermosuccinogenes* 85% -phylotype in the healthy gut could also be a research target. Furthermore, closer investigation of *Gammaproteobacteria* (*Enterobacteria*) phylotypes in IBS could be interesting, and screening of this group in GI samples could be performed with other marker genes than 16S rRNA.

The phylotypes that showed interesting associations with IBS were, however, also found in healthy subjects, and their presence or abundance does not directly indicate IBS. Therefore, it cannot be concluded what is the role of these phylotypes in IBS and whether they are a cause or rather a consequence of altered bowel physiology. This does not mean that observations of this kind on their presence or abundance could not form the basis of a non-invasive marker of the syndrome in the future. Furthermore, assessment of the GI content in IBS could also be extended from microbiota to GI viromes and their dynamics with bacteria, as they are less examined inhabitants in the gut. The IBS-related alterations detected in the GI microbiota in this thesis should definitely be tested and confirmed with other large sample cohorts and extended to mucosal samples. Especially the IBS-D associated microbiota could be a rewarding research target, as it is distinguishable in symptoms and microbiota from other symptom subtypes and healthy individuals. Also, a more detailed parallel study on GI symptoms and microbiota could be carried out in order to define more precise links between these factors. In the future, IBS could potentially be sub-divided also according to other criteria than symptoms.

In order to relieve patients suffering from the symptoms, which can sometimes be severe, use of faecal transplantation in IBS could be considered as a treatment option (Grehan *et al.*, 2010). The method had proven efficiency in CDAD, which shares common microbiota signs but has clear overgrowth of *Clostridium difficile* as a sign of the dysbiosis and a trigger for symptoms (Khoruts *et al.*, 2010). However, probiotic therapy in IBS is more widely acceptable, as it has proven safety and is accessible to all. The ameliorative effect on bowel symptoms and the compositional normalisation the GI microbiota warrants further studies on the mechanisms of action of probiotics in IBS. This could lead to more targeted probiotic therapy of IBS patients suffering from diverse symptoms. Furthermore, the genera used as probiotics could be diversified as our knowledge of the healthy GI microbiota is constantly updated, and *Collinsella aerofaciens* could be one of the candidates.

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